EXHIBIT 8

US005156724A

United States Patent [19]

Jones et al.

[11] Patent Number: 5,156,724

[45] Date of Patent: Oct. 20, 1992

[54] METHOD FOR ANALYZING IONIC SPECIES USING CAPILLARY ELECTROPHORESIS

[75] Inventors: William R. Jones, Blackstone; Petr Jandik, Framingham; Michael Merion, Upton, all of Mass.; Andrea Weston, N. Providence, R.I.

[73] Assignee: Millipore Corporation, Bedford, Mass.

[21] Appl. No.: 642,685

[22] Filed: Jan. 17, 1991

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 471,535, Jan. 29, 1990.

[51] Int. Cl.[5] B01D 57/02; B01D 61/42

[52] U.S. Cl. 204/180.1

[58] Field of Search ..... 204/299 R, 180.1

[56] References Cited

U.S. PATENT DOCUMENTS

4,414,842 11/1983 Small ..... 73/61.1 C
4,936,974 6/1990 Rose 204/180.1

OTHER PUBLICATIONS

Foret, "Indirect Photometric Detection in Capillary Zone Electrophoresis", J. of Chrom., 470 (1989) 299–308.

Foret, "Capillary zone electrophoresis of rare earth metals with indirect UV absorbance detection", (1990) VII, No. 9 pp. 780–783.

Nardi, "Capillary zone electrophoretic separation of cyclodextrins with indirect UV photometric detection", Electrophoresis, 1990, 11, 774–776.

X. Huang et al., *Anal. Chem.*, 61:766–770 (1989).

F. Foret et al., *J. Chromatography*, 470:229–308 (1989).

W. G. Kuhr and E. S. Yeung, *Anal. Chem.*, 60:2642–2646 (1988).

W. G. Kuhr and E. S. Yeung, *Anal. Chem.*, 60:1832–1834 (1988).

T. Takeuchi et al., *Chromatography*, 25:1072–1074 (1988).

K. D. Alria and C. F. Simpson, *Chromatographia*, 24:527–532 (1987).

T. Tsuda et al., *J. Chromatography*, 264:385–392 (1983).

J. W. Jorgenson and K. D. Lukacs, *Science*, 222:266–272 (1983).

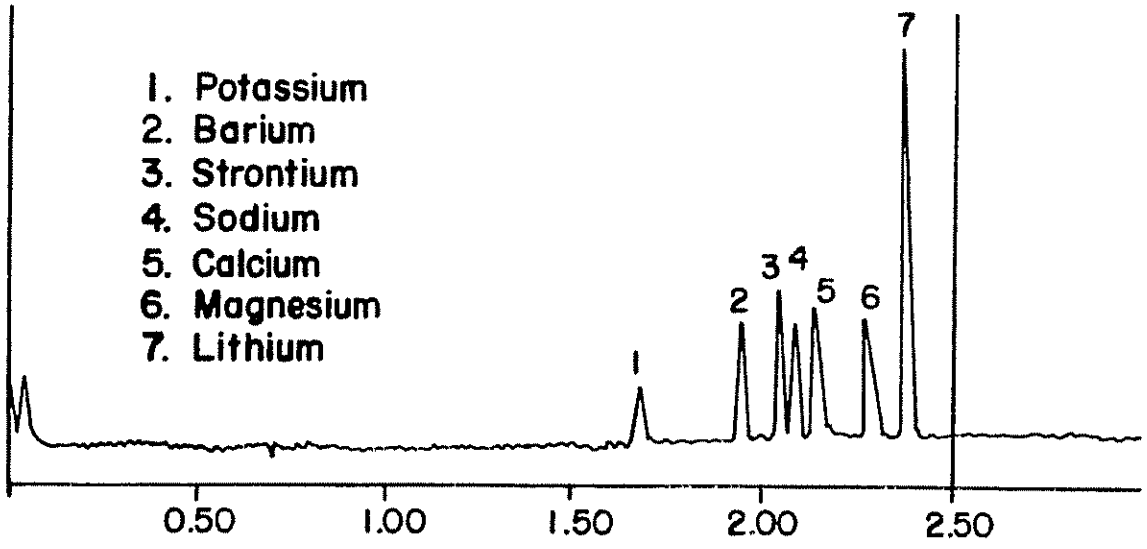
F. Foret et al., *Electrophoresis*, 7:430–432 (1986).

Primary Examiner—John Niebling
Assistant Examiner—Caroline Koestner
Attorney, Agent, or Firm—Hamilton, Brook, Smith & Reynolds

[57] ABSTRACT

A technique for separating, identifying and measuring ions in solution by capillary zone electrophoresis is described, which provides improved sensitivity and resolution of anionic and cationic species. The method involves introducing a sample containing the ionic species into a narrow core capillary filled with a carrier electrolyte containing a selected light-absorbing anion or cation to an electrical current in a capillary column causing the ions to elute according to their ionic mobility. Both UV absorbing and UV-transparent ions can be detected and quantitated by UV/Visible photometric monitoring.

6 Claims, 8 Drawing Sheets



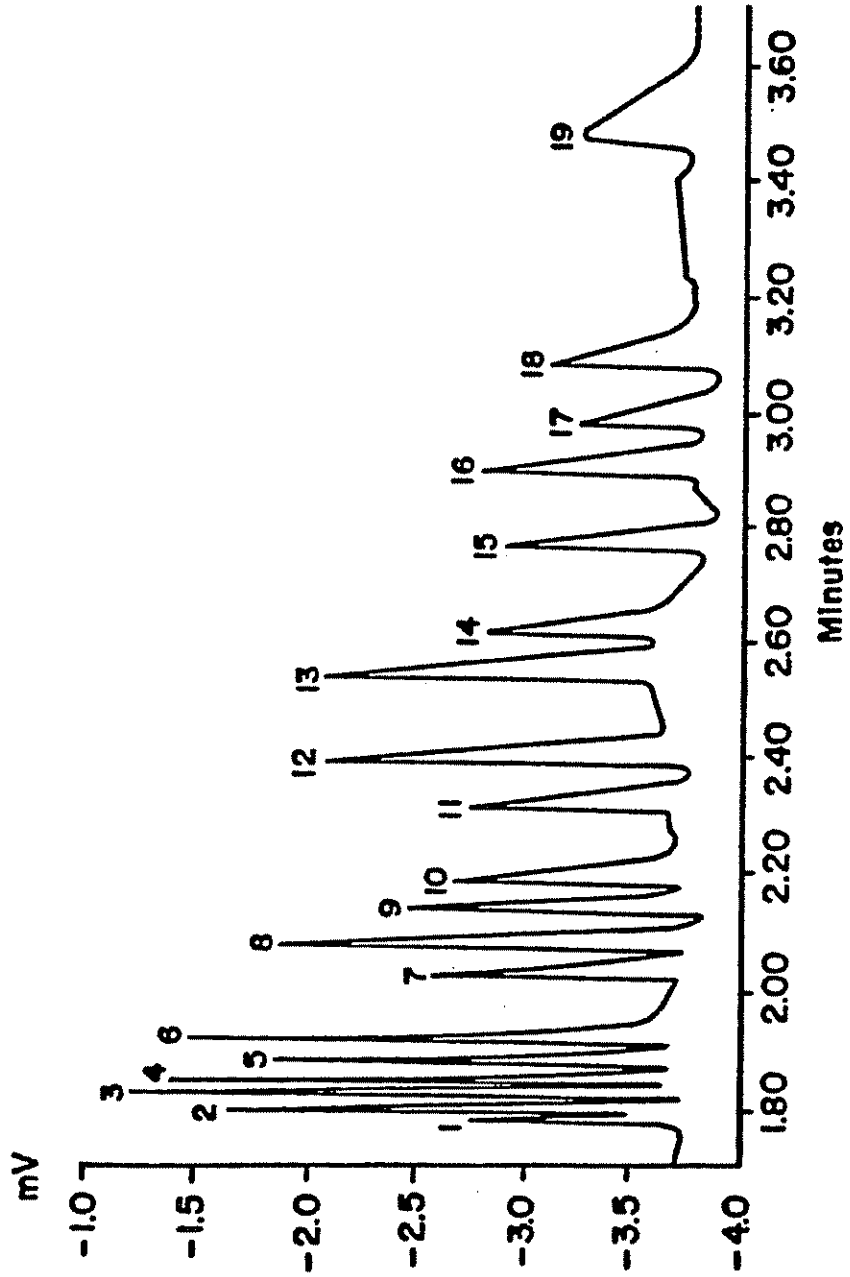

mV
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
1.80
2.00
2.20
2.40
2.60
2.80
3.00
3.20
3.40
3.60
Minutes
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19

FIG. 1

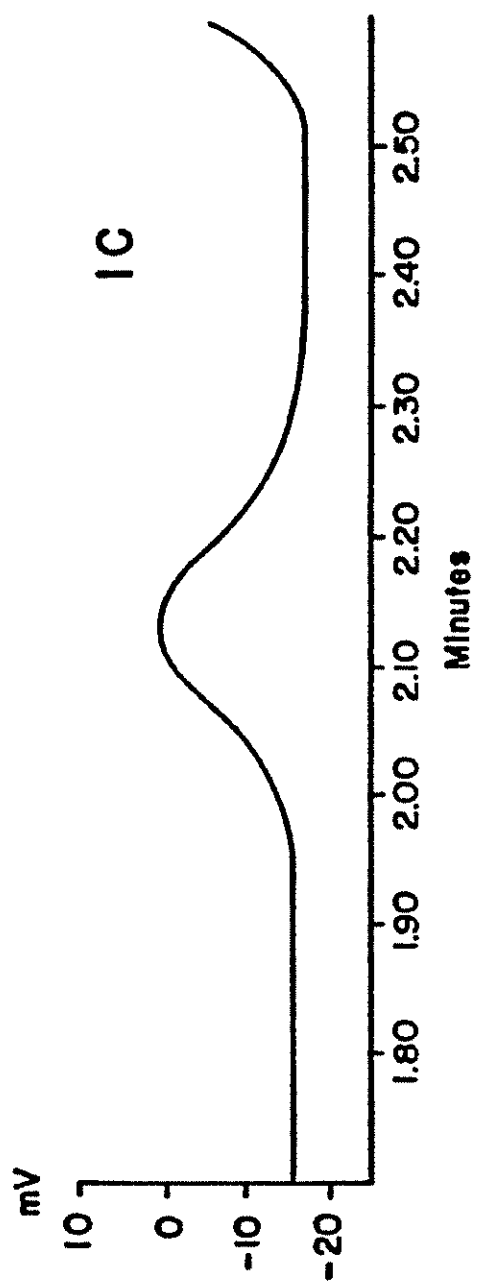

IC
mV
10
0
-10
-20
1.80
1.90
2.00
2.10
2.20
2.30
2.40
2.50
Minutes

FIG. 2A

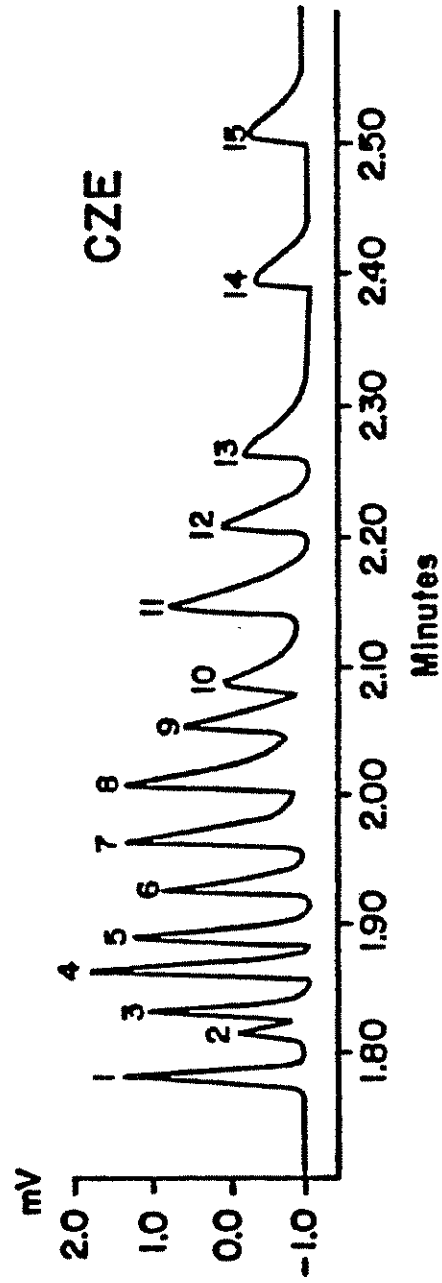

CZE
mV
2.0
1.0
0.0
-1.0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1.80
1.90
2.00
2.10
2.20
2.30
2.40
2.50
Minutes

FIG. 2B

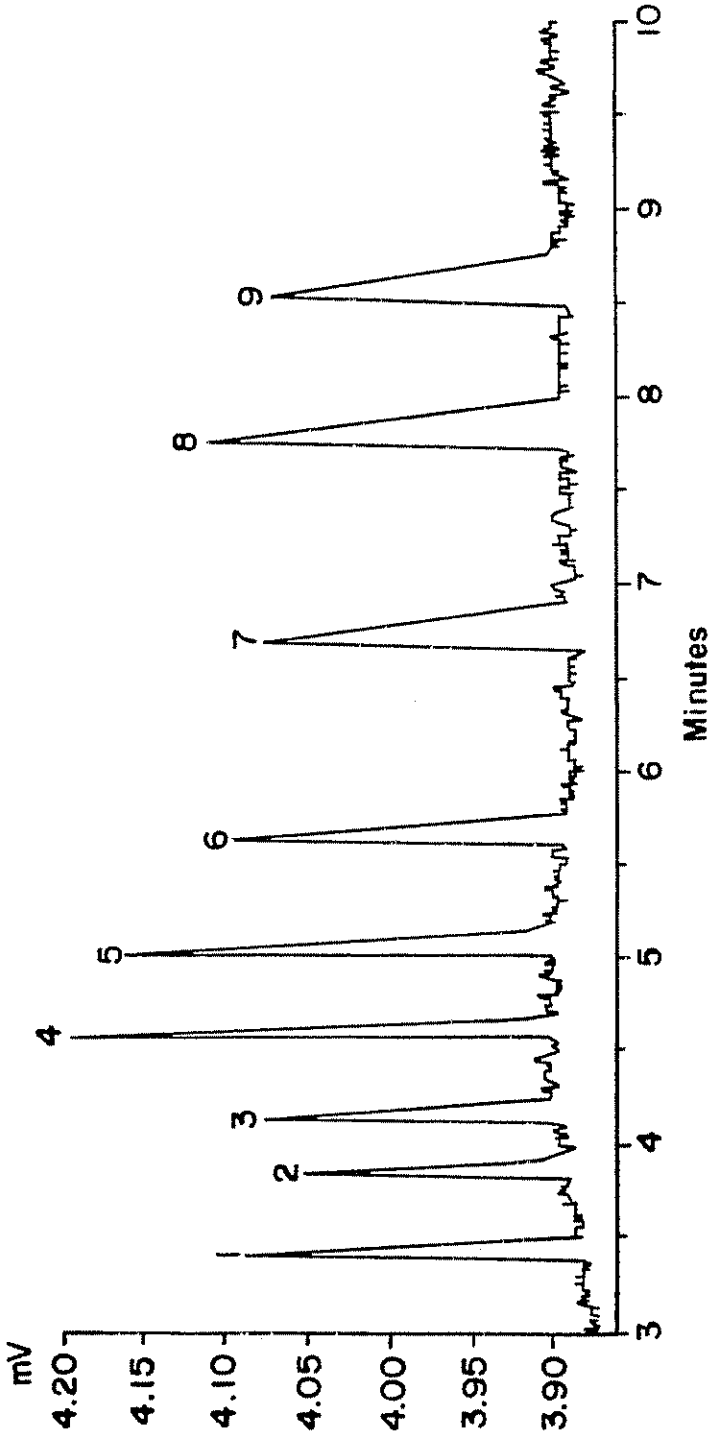


FIG. 3

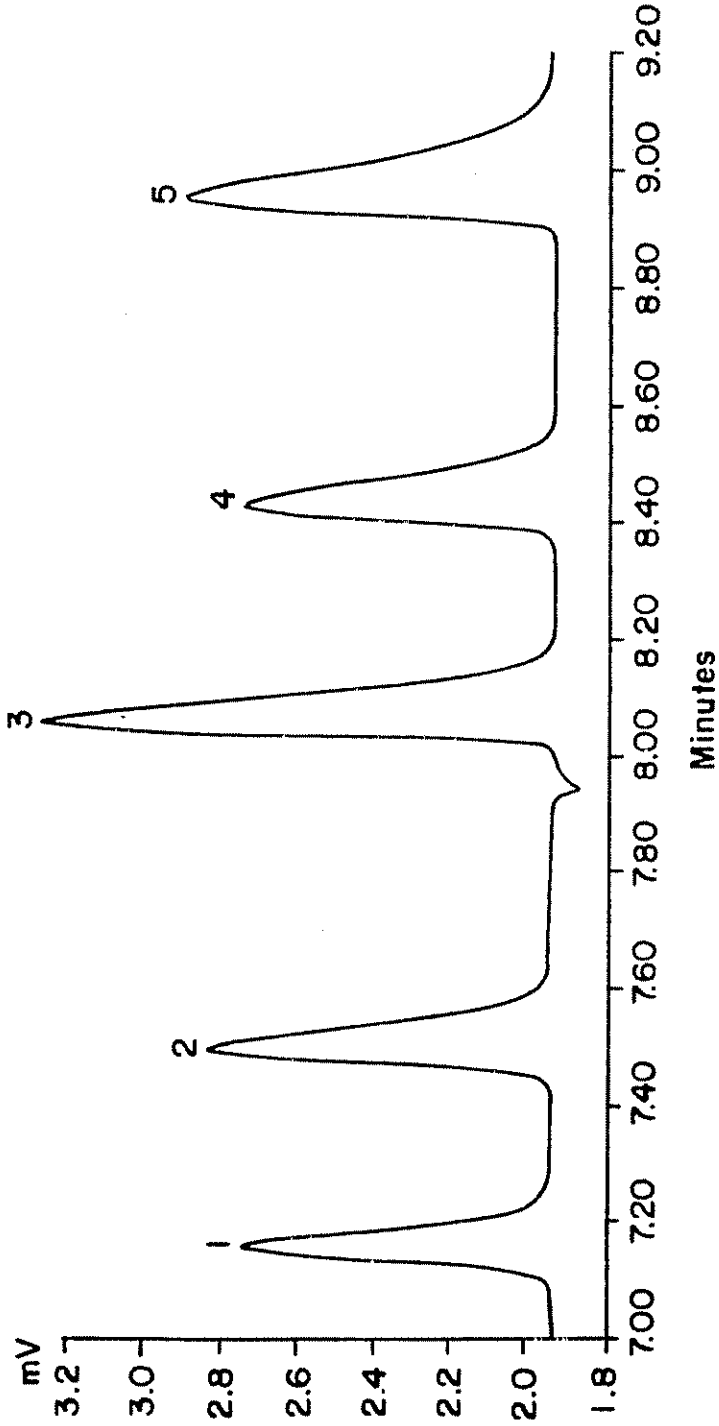

mV
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
7.00
7.20
7.40
7.60
7.80
8.00
8.20
8.40
8.60
8.80
9.00
9.20
Minutes
1
2
3
4
5

FIG. 4

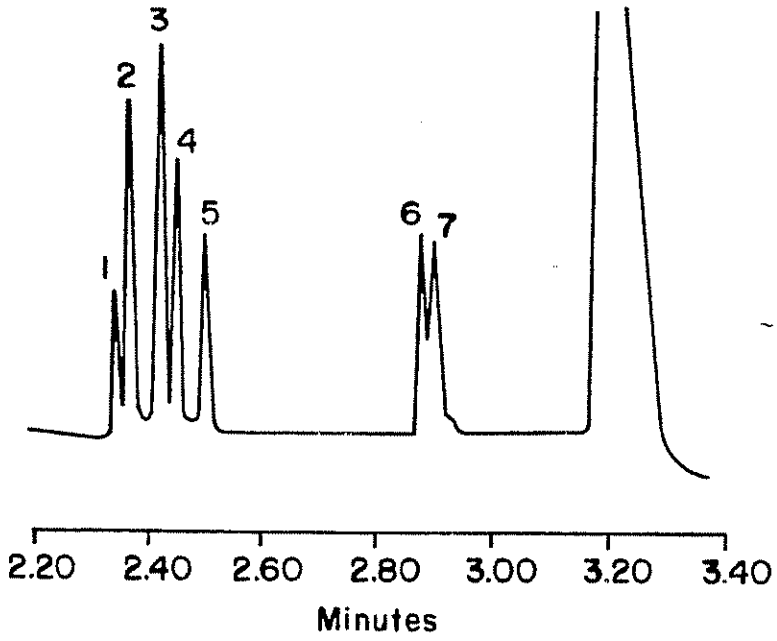


FIG. 5

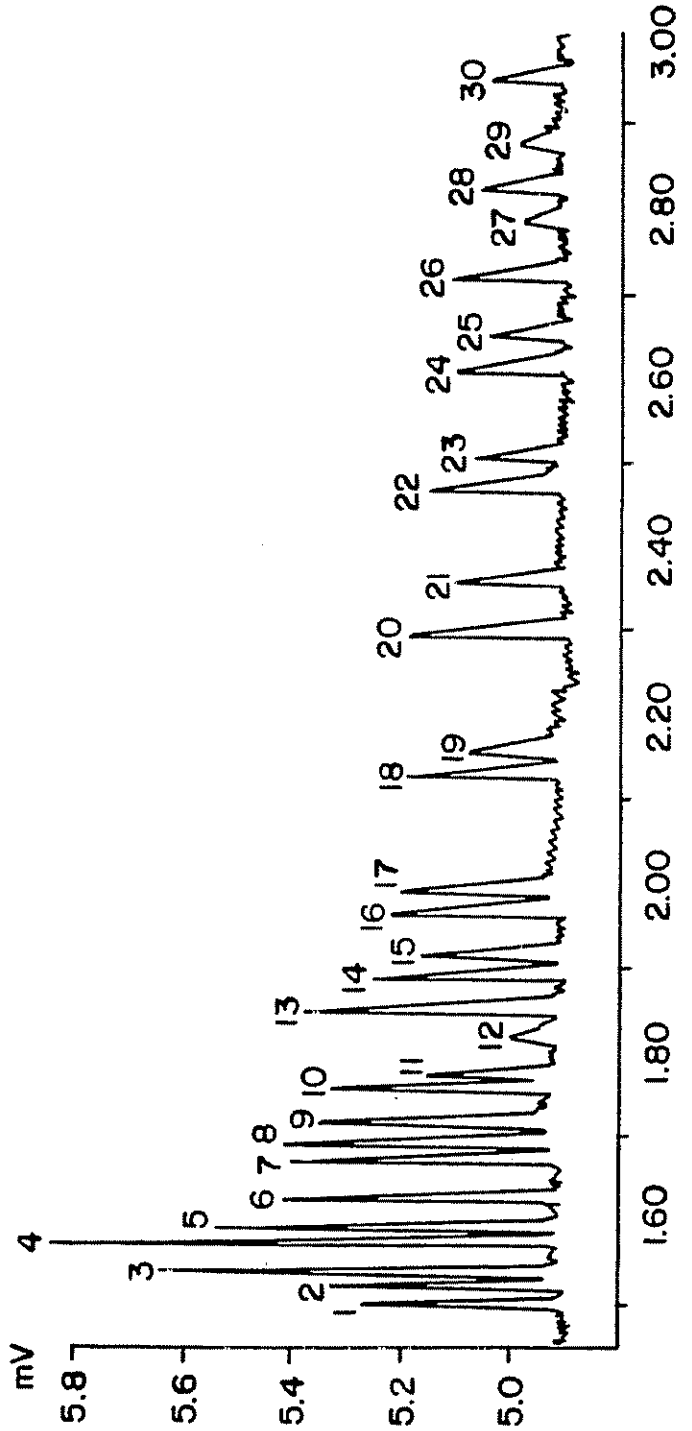

mV
5.8
5.6
5.4
5.2
5.0
1.60
1.80
2.00
2.20
2.40
2.60
2.80
3.00
Minutes
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

FIG. 6

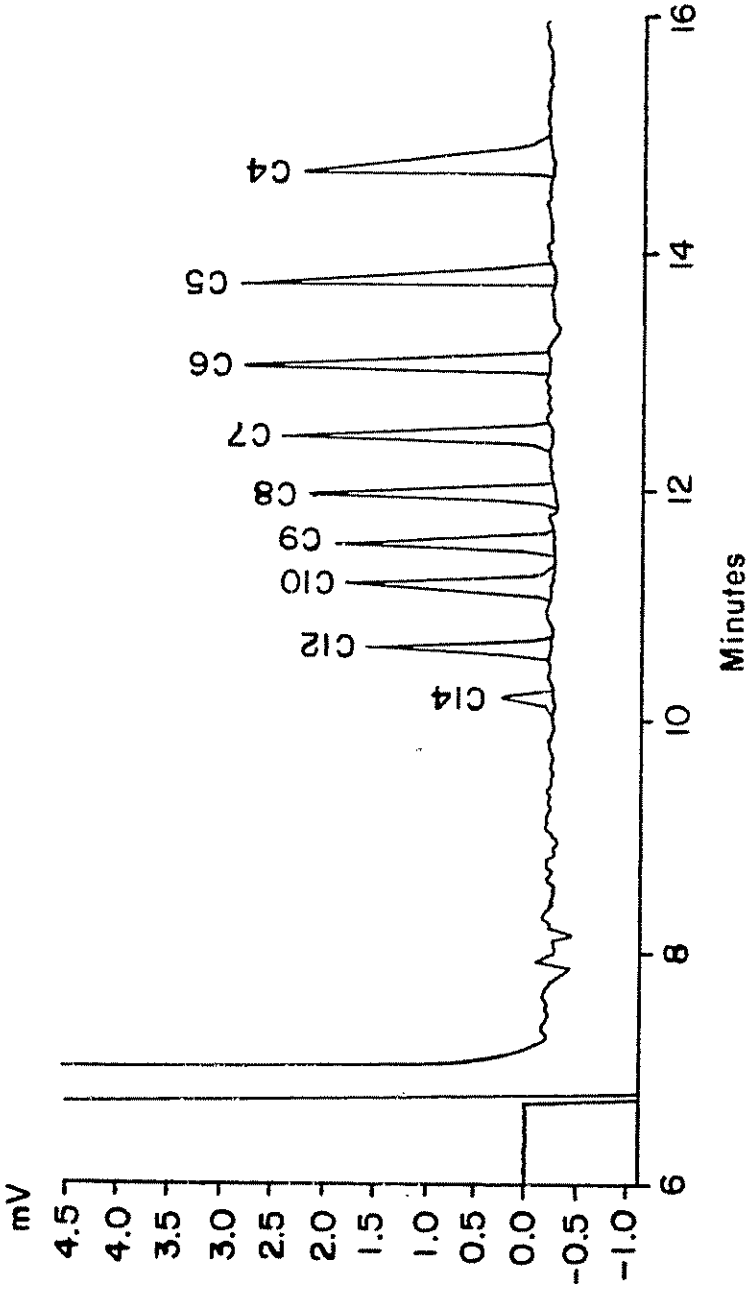

mV
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
6
8
10
12
14
16
Minutes
C4
C5
C6
C7
C8
C9
C10
C12
C14

FIG. 7

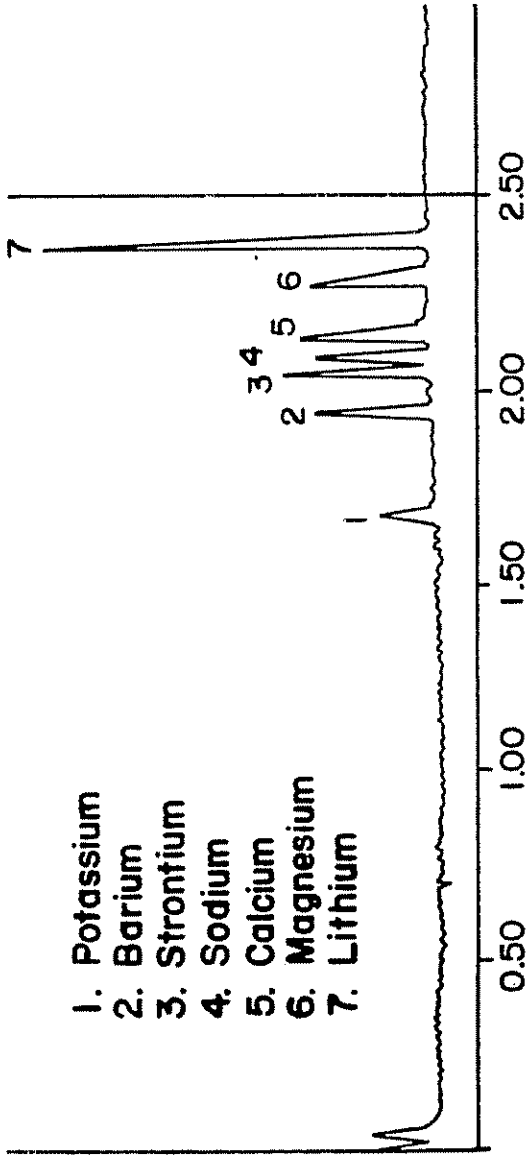

1. Potassium
2. Barium
3. Strontium
4. Sodium
5. Calcium
6. Magnesium
7. Lithium
0.50
1.00
1.50
2.00
2.50
1
2
3
4
5
6
7

FIG. 8

METHOD FOR ANALYZING IONIC SPECIES USING CAPILLARY ELECTROPHORESIS

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Ser. No. 07/471,535, filed Jan. 29, 1990, entitled "Method For Separating Ionic Species Using Capillary Electrophoresis" by William R. Jones, Petr Jandik and Michael Merion.

BACKGROUND

The separation and/or detection of ionic species is generally carried out by utilizing electrochemical properties of analytes, such as ionic interactions and conductivity in ion chromatography or ionic mobility in capillary electrophoresis. Ion chromatography (IC) is capable of detecting simultaneously a large variety of ionic species at low concentration levels. The ability to separate and detect several widely different ionic species simultaneously is a unique characteristic of IC. In fact, the commercial viability of IC depends in part on its ability to simultaneously separate and detect, inter alia, seven common inorganic anions (F^-, Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^- and SO_4^-). However, there are important limitations to IC, including lack of sufficient selectivity for certain types of mixtures, low separation efficiency and a relative complexity of instrumentation.

Capillary electrophoresis (CE) is an efficient analytical separation technique for analysis of minute amounts of sample. CE separations are performed in a narrow diameter capillary tube, which is filled with an electrically conductive medium termed the "carrier electrolyte". A current is applied to the carrier electrolyte, and ionic species in the sample move from one electrode toward the other at a rate which is dependent upon certain characteristics, such as molecular charge, size and/or mobility. CE may be performed using gels or liquids, such as buffers, in the capillary. In the liquid mode, known as free zone electrophoresis, separations are based on the ratio of charge to Stoke's radius.

CE has several advantages over IC and conventional gel electrophoresis for the separation of ionic species. These include improved resolution and smaller sample size. In part, high resolution can be obtained since band broadening is minimized due to the narrow capillary diameter. In free-zone electrophoresis, the phenomenon of electroosmosis, or electroosmotic flow (EOF), which is the bulk flow of liquid rapidly moves all of the sample molecules whether they are positively charged, negatively charged or neutral. Under certain conditions EOF can contribute to improved separation speed in free-zone CE.

The detection of ionic species by CE is problematical particularly if all seven of the common anions mentioned above are to be determined simultaneously. Most ions do not absorb light, so they cannot be detected by conventional photometric means, e.g., direct photometric or fluorescent detection. However, these ions can be detected using indirect photometric detection. Indirect photometric detection relies upon the presence of a light absorbing electrolyte ion in the background electrolyte. Non-absorbing species are detected as zones of decreased absorbance or voids in the background due to the displacement of the light absorbing electrolyte ion. Indirect photometric detection has been described using fluorescent, ultraviolet (UV) and UV-visible (UV-vis) absorbing ions in the background electrolyte. For example, Small et al. in U.S. Pat. No. 4,414,842 describe a technique for detecting ions in an ion exchange chromatography system by indirect UV detection in which a UV-absorbing ion is included in the elution buffer. Methods utilizing indirect photometric detection in capillary electrophoresis have been described by Foret et al., *J. Chromatography*, 470:299-308 (1989); Kuhr et al., *Anal. Chem.*, 60:2642-2646 (1988); Kuhr et al., *Anal. Chem.*, 60:1832-1834. However, these and other methods have not proved satisfactory. For example, none of these methods were able to separate and detect a mixture of eight standard anions (Br^-, Cl^-, SO_4^-, NO_2, NO_3, F^-, HPO_4^- and CO_3^-). The main reason is the inability of previously reported indirect photometric methods to provide the same level of sensitivity for UV transparent ions (e.g., F^-, Cl^-, SO_4^-) and UV-absorbing ions (e.g., NO_2^-, NO_3^-). All published CE methods have failed to successfully separate ions of widely differing properties, e.g., slow migrators such as F^-, PO_4^- and fast migrators such as Br^-, SO_4^-. The need exists for a method for separating and detecting these and other ionic molecules which is faster, more efficient, has better resolution, and requires less sample preparation than the available methods.

SUMMARY OF THE INVENTION

The present invention relates to methods for separating and detecting ions by CE using carrier electrolyte solutions which facilitate detection by indirect methods, particularly UV/visible spectroscopy. The present methods rely upon reagents which can simultaneously effect a sensitive, high resolution separation of several widely different ionic species, ranging from simple inorganic ions to complex organic ions, and both slowly migrating and quickly migrating ions. Methods for separating both anions and cations are disclosed.

The methods generally involve introducing a sample containing the ions into a CE system which utilizes reagents which provide a light-absorbing background at a wavelength suitable for sensitive and interference-free indirect photometric detection of all ionic species without regard to their respective intrinsic UV absorption properties.

The sample is injected into a capillary filled with the carrier electrolyte containing the reagent mixture, an electric current is applied to the capillary under conditions appropriate to cause the ions in the mixture to move toward the oppositely charged electrode and the ionic species are detected photometrically.

The reagent mixture which is most effective as a component in a carrier electrolyte for separating anions consists of the salt of a UV-absorbing anion (e.g., iodide, tungstate, molybdate, chromate, ferrocyanide, ferricyanide or vanadate). Chromate and vanadate compounds are preferred reagents for most anion separations, in part because of their ionic mobilities relative to the common inorganic anions and because of their unusually broad UV spectra. In addition, one or more reagents for controlling the speed and/or direction of the electroosmotic flow of the carrier electrolyte can, optionally, be included in the electrolyte mixture. For example, an alkyl quaternary ammonium, phosphonium or arsonium salt having at least eight carbon atoms in a linear or branched configuration can be added. Sodium chromate is a particularly preferred UV-absorbing salt and tetradecyltrimethylammonium bromide (TTAB) or cetyltrimethylammonium bromide (CTAB) are particu-

larly preferred flow modifiers. Alternatively, the carrier electrolyte can contain only the salt of a UV-absorbing anion while the ammonium, arsonium or phosphonium groups are bound (chemically or by absorptive forces) to the capillary wall.

In addition to the UV-absorbing anion and the flow modifier, an electromigrative agent can be added to the system. The electromigrative agent which enhances the detection of trace anions, e.g., species present in nanomole concentrations and is generally added to the sample containing the analyte ions.

A reagent composition which is an effective carrier electrolyte for separating cations is also the subject of the present invention. The reagent is selected to allow separation and detection of cations having widely different properties (e.g., alkali and alkaline earth cations in a mixture with transition metals). This reagent composition consists of a UV absorbing amine, such as 4-methylbenzylamine, heterocyclic compounds with or without sulfonic groups, such as, for example, 2[N-morpholino] ethanesulfonic acid (MES) or naphthalene sulfonic acid, alkyl or aryl sulfonic acids, with or without additional UV absorbing groups, such as, for example, dodecylfonic acid. The carrier electrolyte can, optionally, also contain one or more chelating or complexing agents. The chelating or complexing agents are particularly useful for separating cations having the same or very close mobility.

The chemistry necessary to perform CE separations of ionic species for indirect detection can be contained in a kit. Such a kit for separating anions would contain, inter alia, one or more light absorbing ions specific for the UV/visible range, such as a chromate and/or vanadate salt, and optionally, a quaternary ammonium, arsonium or phosphonium compound. For detecting cations, the kit would contain a UV absorbing cation, such as 4-methylbenzylamine or MES, and optionally, one or more complexing or chelating agents, which are added to the sample in cases where groups of cations having approximately the same mobilities must be separated.

The present reagent compositions and methods have several advantages, such as improved sensitivity, linearity of the range of calibration, the ability to separate and resolve a wide range of anionic and cationic species, the ability to detect ionic species which are not detectable by direct methods in addition to ions that are detectable by direct methods, less sample preparation and faster separation. The methods can be used to separate and detect both simple and complex anions or cations, and to detect a variety of analytes simultaneously.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a chromatogram showing the separation of nineteen anions by CE using TTAB/Na_2CrO_4 as the carrier electrolyte.

FIG. 2 compares (A) a chromatogram showing the separation of fifteen anions using IC; with (B) a chromatogram showing separation of the same mixture of anions by CE using TTAB/Na_2CrO_4 as the carrier electrolyte.

FIG. 3 is a chromatogram showing the separation of nine anions by CE using Na_2CrO_4 as the carrier electrolyte and modifying the capillary walls with an uncharged polymer, effectively shielding the silanol groups on the walls.

FIG. 4 is a chromatogram showing the separation of five anions by CE using Na_2CrO_4 and an amphoteric flow modifier as the carrier electrolyte.

FIG. 5 is a chromatogram showing the separation of eight anions by CE using TTAB/Na_2CrO_4 and octanesulfonate as an electromigrative agent.

FIG. 6 is a chromatogram showing the separation of thirty anions by CE using TTAB/Na_2CrO_4 and electromigrative sample injection.

FIG. 7 is a chromatogram showing the separation of nine anions by CE using naphthalene sulfonate as the electrolyte.

FIG. 8 is a chromatogram showing the separation of seven cations by CE using 4-methylbenzylamine as the carrier electrolyte.

DETAILED DESCRIPTION OF THE INVENTION

The present method utilizes CE to simultaneously separate and detect ionic species having widely different properties contained in a sample using indirect UV/visible detection. Indirect UV/visible spectroscopy is used because many ionic species cannot be detected using direct detection methods. CE is a well known technology, and has been described in detail, for example, by Compton and Brownlee in *Biotechniques*, 6(5): 432–440 (1988); and Jorgenson and Lukacs in *Science*, 222:266–272 (1983). A method of utilizing indirect photometric detection in CE is described by Foret et al. in *J. Chromatography*, 470:299–308 (1989).

The present method is generally carried out using the following procedure: a capillary tube is filled with an electrically conductive liquid (the carrier electrolyte) containing one or more reagents which facilitate detection by UV/visible spectroscopy. A preferred capillary is generally a fused silica capillary having an internal diameter of from about 50 to 100 microns (μ).

The ionic sample is introduced into the capillary, for example, by hydrostatic pressure, vacuum or by electromigrative injection in which the liquid sample is moved into the capillary by an electric current. After introduction of the sample, each end of the capillary is immersed in a reservoir which contains an electrode and the carrier electrolyte solution containing the reagents. The capillary tube is positioned with a detector on the column near the end opposite to sample introduction. Electric current is applied causing the anions or cations to move along the capillary toward the opposite electrode. The ions move at different speeds, depending upon several factors, such as their size and mobility. The electrophoretic separation is preferably monitored by indirect UV/visible spectroscopy. Other indirect detection methods can be used, however, UV/visible spectroscopy is preferred because it allows sensitive rapid detection of ionic species and is less costly than laser enhanced fluorescence detection, for example.

The method relies upon reagents which facilitate detection by indirect UV/visible spectroscopy, comprising light-absorbing compounds specific for the UV/visible range. For detecting anions, a UV-absorbing anion is used and for detecting cations, a UV-absorbing cation is used in the carrier electrolyte.

UV/visible light-absorbing compounds which are useful for separating and detecting anions are UV-absorbing anions, such as iodide, tungstate, molybdate, chromate, ferrocyanide, ferrocyanate and vanadate salts. Absorbing anions which are particularly useful are selected chromate and vanadate salts. A preferred chromate salt is sodium chromate (Na_2CrO_4) having a concentration of from about 1 mM to about 20 mM. A

preferred vanadate salt is sodium vanadate having a concentration of from about 1 mM to about 10 mM.

The carrier electrolyte can also contain, in addition to the UV-absorbing anion, a flow modifier, which is a compound which slows, stops or reverses the electroosmotic flow of the carrier electrolyte. Electroosmotic flow is the bulk flow of the electrolyte through a capillary that is induced by an applied electric field. The amount of flow and its direction is dependent on the charge of the inner wall of the capillary. If there is no wall charge, there is no electroosmotic flow. Thus, flow modifier can eliminate or reverse the effects of the capillary wall on the flow of the electrolyte. Negating or counteracting the wall effects can improve the resolution of the desired analyte ions. Flow modifiers which are useful in the present method include cationic surface active agents, such as alkyl ammonium, arsonium and phosphonium compounds containing at least eight carbon atoms in a linear or branched configuration. Such compounds include for example, quaternary ammonium salts, arsonium salts and phosphonium salts, biammonium salts, biphosphonium salts and biarsonium salts. These compounds include, for example, octyl trimethylammonium, phosphonium or arsonium, various alkyl derivatives of 1,8-diaminooctane, 1,8-diphosphinooctane or 1,8-diarsinooctane. Also suitable are some polymeric ammonium, phosphonium and arsonium salts, such as, for example, hexadimethine bromide. Amphoteric ammonium compounds, such as, for example 3(N,N-dimethylpalmityl-ammonio)propanesulfonate are also useful flow modifiers. Compounds which are particularly useful are quaternary ammonium salts which contain alkyl groups having at least eight carbon atoms in a linear or branched configuration. Preferred quaternary ammonium salts are tetradecyltrimethylammonium bromide (TTAB) and/or cetyltrimethylammonium bromide (CTAB). A concentration of TTAB or CTAB of from about 0.1 mM to about 5.0 mM is useful in the present method. The use of flow modifiers facilitates control of both the direction, as well as the rate of electroosmotic flow. Control of this parameter permits the development of an assay that is both high in resolution and is complete in a short period of time. The carrier electrolyte solution generally has a pH of from about 7.5 to about 8.5. An acid, such as sulfuric acid or chromic acid, can be added to the electrolyte solution to adjust the pH to the desired level.

In another embodiment of the present method, various aromatic carboxylic acids can be used as components in carrier electrolytes. The main usefulness of carboxylates as carrier electrolytes is in the CE analysis of less mobile anions (e.g., fluoride, carboxylic acids, alkylsulfonates), which may produce broadly asymmetric peaks if analyzed using the chromate electrolyte. However, because of their relatively low mobilities, aromatic carboxylates are less suitable than chromates as electrolytes for analysis of complex mixtures of highly mobile inorganic anions. The best results are obtained in CE when the mobilities between the main anionic components of the carrier electrolyte and the analyte ions is closely matched. Therefore, a range of highly UV-absorbing carrier electrolytes covering the range of ionic mobilities of all inorganic anions and other low molecular weight species is of practical interest. Aromatic carboxylates are useful in the present method for detecting and measuring some organic anionic species having low ionic mobility and which are UV-transparent, such as carboxylic acids, amino acids, carbohydrates or sulfonates. Aromatic carboxylates such as phthalate, trimesate, benzenetetracrboxylate, p-hydroxybenzoate, and p-anisate are useful for this purpose.

In another embodiment of the present composition and method for separating and detecting anions, an electromigrative agent can be added to the sample in order to enhance the separation and/or detection of trace amounts of anions, e.g. nanomolar quantities. The addition of an electromigrative agent to the sample provides enrichment of the separation of anions present in the sample in concentrations in the nanomolar range. In this embodiment, the agent is added to the sample, and the sample is injected into the capillary using electromigrative sample introduction. Electromigrative sample introduction involves applying a current having very low amperage which selectively causes the trace anions to migrate toward the capillary. The addition to the sample of the electromigrative agent, which has a lower ionic mobility in comparison to the carrier electrolyte anion, results in the selective migration of the trace anions into the capillary, which effectively preconcentrates these anions, thereby enriching the sample to be analyzed with the trace anions. In addition to the analyte anions, it is also possible to observe the enrichment of sample matrix anions acting as an isotachophoretic terminating electrolyte. Such anions may be added purposely. In solutions containing total ionic concentrations in the nanomolar range, sample conductivity often becomes too low, and can be adjusted by a suitable additive to enable sufficient electric charge throughput for ionic transfer from the bulk of the sample solution into the capillary. For this purpose, citrate, carbonate and octanesulfonate salts can be used as electromigration additives.

Citrate, carbonate and octanesulfonate salts which exhibit lower ionic mobilities in comparison with the UV-absorbing anion in the carrier electrolyte (e.g., chromate) can be used as additives for electromigrative trace enrichment with the UV-absorbing anions in the carrier electrolyte. Sodium octanesulfonate adjusted within the range of 15 to 40 μm is particularly useful electromigration agent in low ionic content samples. Addition of relatively excessive concentrations of octanesulfonate does not lead to interfering comigration with any of over fifty anionic species analyzed by the present CE method. Sodium octanesulfonate can be obtained free of common ionic impurities which could disturb the quantitation of common anions such as sulfate and chloride in unknown samples. An example of a separation of common inorganic anions at low ppb levels using sodium octanesulfonate as an electromigrative agent is shown in FIG. 5. As indicated, the detection limits (calculated as noise times three in concentration units) for this separation are in the low nanomolar range. This represents at least a hundredfold increase in sensitivity in comparison with the results achievable in the same carrier electrolyte and with hydrodynamic sample introduction.

UV/visible light-absorbing compounds which are useful in the present method for separating and detecting cations are UV-absorbing cations, such as 4-methylbenzylamine, 2-aminopyridine, 2-amino-4,6-dimethylpyridine, MES, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and N-[2-hydroxyethyl]piperazine-N'-[3-propane sulfonic acid] (EPPS). 4-Methylbenzylamine is the preferred UV-absorbing cation in the present method. The concentra-

tion of 4-methylbenzylamine is generally from about 3 mM to about 10 mM. The reagent mixture for cation separation can optionally contain a complexing or chelating agent, such as ethylenediaminetetraacetic acid (EDTA), citrate, tartrate, hydroxyisobutyrate, oxalate and succinate. The complexing agent allows cations having the same or similar mobilities to be differentiated.

The present methods can be utilized to analyze most types of ionic species. Samples containing complex mixtures of ions, including anions, cations and organic compounds, for example can be analyzed using the method. When a sample containing such a complex mixture is separated using the present methods and electrolyte carriers for separating anions, for example, the detector is placed just before the anode immersed in an electrolyte and the cathode is placed in another portion of the same electrolyte at the opposite end. Thus, the cations in the sample will move away from the detector, and the organic species will move very slowly toward the anode, thereby creating a window for the anionic species toward the detector. The anions move most rapidly toward the detector, thus are most efficiently resolved. Where a method and electrolyte carrier appropriate for separating cations is used, the polarity of the electrodes is reversed, and the cations will move toward the detector (i.e., toward the cathode) while the anions in the sample will move away from the detector.

The present methods are useful for analyzing samples containing multiple ionic species in the shortest time possible, or to scan an unknown sample for ionic compounds, since the methods and reagent mixtures can efficiently separate and resolve such mixtures. Samples which can be analyzed using the present methods include water, foods, such as juices, biological fluids or industrial chemical mixtures.

In one embodiment of the present method, a sample containing eight common inorganic anions: bromide, chloride, nitrate, nitrite, sulfate, fluoride, phosphate and carbonate, was analyzed by CE using a mixture of 0.5 mM TTAB and 5 mM sodium chromate (Na_2CrO_4) having a pH of 8 as the carrier electrolyte. All eight anions were detected by monitoring the absorbance of the carrier electrolyte at 254 or 272 nm. Separation of all eight anions was completed in about three minutes. The ionic species were separated based on their ionic mobilites. This is important because the elution sequence using the present method is predictable based on the known ion mobilites of various ions. This means that the chemical identity of an unknown analyte can be reliably determined from its position in the elution order.

In another embodiment of the present method, a sample containing seven inorganic cations: potassium, barium, strontium, sodium, calcium, magnesium and lithium, was analyzed by CE using 5 mM 4-methylbenzylamine in 0.21 mM citrate having a pH of 5.5 as a carrier electrolyte. All seven cations were detected by monitoring the absorbance of the carrier electrolyte at 214 mM. Separation of all seven cations was complete in less than three minutes.

Separation of ionic species using the present compositions and methods is superior to ion chromatographic separations of similar mixtures in several respects: improved separation efficiency, shorter runtime, better selectivity, linearity of the plot and improved sensitivity. For example, the number of theoretical plates for sulfate in the illustrative example used above is 157,344. The highest plate-counts attainable by ion chromatography are smaller than 10,000. Separation of the standard eight anions was completed in three minutes by the present method, whereas ion chromatographic separations of identical mixtures take typically six to fifteen minutes. Injection volumes for the CE separation are less than about 40 nanoliters (nl) compared to about 50 to 100 microliters (μl) for IC. Even though only 20 nL were injected to obtain the above separation, detection limits for all separated anions were either comparable or better than those observed in IC. This corresponds to a 10,000 fold increase in absolute sensitivity (per μg injected) in the present CE system in comparison with IC.

The present methods provide ionic separations which are efficient, highly selective, and which have a predictable order of elution. The methods exhibit increased selectivity for ionic separations as compared to other methods such as IC. During a typical CE separation of anions using the present chromate reagent mixture, cationic compounds migrate in the opposite direction away from the anions of interest and are not seen in the electropherogram. Conversely, during a typical CE separation of cations using the methylbenzylamine reagent mixture, anionic compounds migrate in the opposite direction away from the cations of interest. Neutral and slightly polar impurities are considerably less mobile than the anions or cations and have longer migration times. Thus, the anions or cations of interest are efficiently separated and resolved in the shortest time.

The practical usefulness of such increased selectivity can be illustrated, for example, using a fruit juice as the sample. When orange juice is directly injected into an IC system, the first five peaks to elute, which represent fluoride, chloride, nitrite, bromide and nitrate ions, are subjected to interference by carboxylic acids, such as formate or acetate and other organic compounds in the sample. To reduce this interference, analysis of the anions in the juice using IC would require a complicated pretreatment of the sample to remove the carboxylates and organic compounds. The same sample can be successfully analyzed by CE, and good separation of the anions can be obtained without any pretreatment of the sample using the present method.

The invention is further illustrated by the following Examples.

EXAMPLE 1

General Procedure For CZE of Anions Using Na_2CrO_4/TTAB Electrolyte

A sample containing the following eight inorganic anions was prepared: fluoride (F), carbonate (CO_3), chloride (Cl), nitrite (NO_2), nitrate (NO_3), bromide (Br), phosphate (H_2PO_4) and sulfate (SO_2).

A fused silica capillary externally coated with polyimide (Polymicro Technologies) was freshly cut from a roll and approximately 1 cm section of polyimide coating was burned off with a butane lighter for UV to pass through at 40.5 cm from one end. The total capillary length was 63 cm, and had an internal diameter of 75 μm. The capillary was installed into the cell and purged with electrolyte with a 1 cc luer syringe with an adapter. The electrolyte was 5 mM Na_2CrO_4 and 0.5 mM TTAB, adjusted to pH 8 with 10 mN sulfuric acid. A 50 ml beaker and a 100 ml beaker were filled with electrolyte to equal heights. The 50 ml beaker was placed at the cathode end of the capillary and the 100

ml was placed at the anode end. Approximately 100 microliters of carrier electrolyte was run through the capillary prior to analysis.

The power supply (Spellman (0 to 30 KV)) was manually turned to zero. The capillary at the cathode end was picked up manually, raised to 16 cm height above the electrolyte level and placed in the sample for 30 seconds. The capillary was removed from the sample and placed promptly into the electrolyte. The voltage was manually ramped from 0 to 20 KV during approximately 10 seconds while the start integrate signal was initiated at the beginning of the voltage ramp. At 20 KV a typical current reading was about 20 μA.

Detection was carried out using a Linear Instruments variable UV/Vis CE detector at two different wavelengths: 254 nm and 272 nm.

Separation was completed in about three minutes, and a clear and distinct peak was obtained for each anion. All eight anions were separated within about one minute.

EXAMPLE 2

CE Separation of a Complex Mixture of Anions

The separation of a complex mixture of ten (10) weakly and strongly dissociated anionic species was carried out according to the procedure described in Example 1. The ten anions in the mixture were: Cl, SO_4, NO_3, F, CO_3, formate, acetate, propionate, butyrate and an unidentified organic acid. Separation was completed within about 3.8 minutes. All ten anions eluted and were detected, and a clear and distinct peak was obtained for each anion.

EXAMPLE 3

CE Separation of a Complex Mixture of Nineteen Anions

The separation of a complex mixture of nineteen anionic species was carried out according to the general procedure described in Example 1. The injection volume was 20 nl, and indirect UV/visible detection was carried out at 272 nm. The nineteen anions were:

	anion	ppm
1.	bromide	4
2.	chloride	2
3.	sulfate	4
4.	nitrite	4
5.	nitrate	4
6.	molybdate	20
7.	citrate	4
8.	fluoride	1
9.	phosphate	4
10.	phoshite	4
11.	phthalate	4
12.	methanesulfonate	5
13.	ethane sulfonate	5
14.	acetate	5
15.	propanesulfonate	5
16.	butane sulfonate	5
17.	benzoate	5
18.	pentane sulfonate	5
19.	hexane sulfonate	5

Separation was completed in less than four minutes. All nineteen anions were detected and a clear and distinct peak was obtained for each anion, as shown in FIG. 1. The numbers on the peaks correspond to the numbers in the above list of anions.

EXAMPLE 4

Comparison of a CE Separation and an IC Separation of Fifteen Anions

The separation of a mixture of 15 anions was carried out by CE according to the procedure set out in Example 3. The same mixture was separated by IC according to standard IC conditions. The fifteen anions were:

	anion	ppm
1.	thiosulfate	4
2.	bromide	2
3.	chloride	2
4.	sulfate	4
5.	nitrite	4
6.	nitrate	4
7.	molybdate	20
8.	tungstate	20
9.	monofluorophosphate	4
10.	citrate	4
11.	fluoride	1
12.	phosphate	4
13.	phosphite	4
14.	phthalate	4
15.	carbonate	4

The results are shown in FIG. 2. FIG. 2A is a chromatogram of the IC results after 2.5 minutes. The large rounded peak represents the carbonate ion (HCO_3^-), and the curve which starts upward at about the 2.5 minute mark represents the start of the chloride ion (Cl^-) peak.

FIG. 2B is a chromatogram showing the CE separation. Separation of all fifteen anions was completed in about 2.5 minutes, and a clear and distinct peak was obtained for each anion.

The results showed that for identical ppm levels of each anion, approximately the same signal to noise ratios were observed by CE from an injection volume of 20 nL as by IC for an injection volume of 100 μl. These results indicate that the CE method is about 5000 times more sensitive than conventional IC. In this example, it took about two minutes for an average IC peak to elute under standard conditions wherein the CE method separated fifteen peaks in the same period of time. The observed increase in sensitivity is due to increased separation efficiency: about 1000 theoretical plates for IC vs about 100,000 for CZE.

EXAMPLE 5

CE Separation of a Mixture of Nine Anions Using a Modified Capillary

The separation of a mixture of nine anions was carried out according to the procedure described in Example 1, except that no flow modifier (TTAB) was used. The capillary wall was modified by covering the inner wall with a layer of PSDVB polymer to shield the negative charges of the silanol groups present on the wall. The capillary was 46 cm in length and had an internal diameter of 50 μm. All nine anions were separated by the procedure in less than nine minutes, as shown in FIG. 3. The anions shown in FIG. 3 are:

1. thiosulfate
2. bromide
3. chloride
4. sulfate
5. nitrite
6. nitrate

7 molybdate
8 azide
9 tungstate.

EXAMPLE 6

CE Separation of Five Anions Using An Amphoteric Flow Modifier

Separation of a mixture of five anions was carried out according to the procedure described in Example 1, except that an amphoteric detergent, 3(N,N-dimethylpalmitylammonio)propanesulfonate (pH 8, 0.5 mM), was used in lieu of TTAB. The capillary was 60 cm in length and had an internal diameter of 75 μm. All five anions were separated in about nine minutes, as shown in FIG. 4. The anions shown in FIG. 4 are:

1. Br
2. Cl
3. SO_4
4. NO_2
5. NO_3.

EXAMPLE 7

Inproving Sensitivity of Separation of an Eight Anion Mixture by Electromigrative Sample Introduction

CE separation of an eight anion mixture was carried out as described in Example 1 above, except that an electromigration enhancer sodium octanesulfonate, was added to enhance sensitivity for trace amounts of anions.

The carrier electrolyte contained 5 mM chromate and 0.5 mM TTAB electroosmotic flow modifier and was adjusted to pH 8.1. Fused silica capillary (75 μm internal diameter, 52 cm from the point of sample introduction to the detector) was used for the separation. During the analysis, the injection side was at −20 kV. The electromigrative sample introduction was carried out at 5 kV for 45 seconds. Sample conductivity was adjusted by the addition of sodium octanesulfonate at 18 μN to the sample. The peak identities, ppb concentrations and nM detection limits (3× the noise), shown in FIG. 5, were as follows: Peak 1: Bromide 4 ppb, 13.6 nM; 2: Chloride 4 ppb, 13 nM; 3:Sulfate 4 ppb, 8.4 nM, 4:Nitrite 4 ppb, 25.4 nM; 5:Nitrate 4 ppb, 24 nM; 6:Fluoride 2 ppb, 19.8 nM and 7-Phosphate 8 ppbm 17.8 nM. The large peak at about 3.2 minutes is the carbonate. The levels of carbonate were not controlled under the conditions of this experiment. These results show that the detection limits (calculated as 3× noise in concentration units) for this separation are in the low nanomolar range, which represents at least a hundredfold increase in sensitivity in comparison with the results achievable in the same carrier electrolyte without the addition of sodium octanesulfonate to the sample.

EXAMPLE 8

CE Separation of a Thirty Anion Mixture Using Electromigrative Sample Introduction

CE separation of a thirty anion mixture was carried out as described in Example 7, using electromigrative sample introduction. The electromigrative sample introduction was carried out at 1 KV for 14 seconds. The capillary was 60 cm in length and had an internal diameter of 50 μm. The electrolyte was 5 mM Na_2CrO_4 and 0.5 mM TTAB, pH 8.0. All thirty anions were separated in less than three minutes, as shown in FIG. 6. The anions shown in FIG. 6 are listed below:

1. thiosulfate
2. bromide
3. chloride
4. sulfate
5. nitrite
6. nitrate
7. molybdate
8. azine
9. tungstate
10. monofluorophosphate
11. chlorate
12. citrate
13. fluoride
14. formate
15. phosphate
16. phosphite
17. chlorite
18. galactarate
19. carbonate
20. acetate
21. ethanesulfonate
22. propionate
23. propanesulfonate
24. butyrate
25. butanesulfonate
26. valerate
27. benzoate
28. glutamate
29. pentanesulfonate
30. gluconate.

EXAMPLE 9

CE Separation of Nine Anions Using Naphthalene Sulfonic Acid Electrolyte

A mixture of nine anions was separated according to the procedure described in Example 1. The nine anions, designated C4–C10, C12 and C14, are linear alkylsulfonates having from 4 to 14 carbon atoms, at a concentration of 25 ppm each. A capillary 60 cm long and having an internal diameter of 75 μm was used. The electrolyte was 10 mM naphthalene sulfonic acid (30% ACN) adjusted to pH 10 with NaOH. The sample was injected by hydrostatic injection for 30 seconds. Indirect UV detection was used at a wavelength of 254 nm.

The results are shown in FIG. 7. All nine anions were separated and elution was complete in about fifteen minutes.

EXAMPLE 10

General Procedure for CE Separation of Cations Using 4-Methylbenzylamine Electrolyte

A sample containing the following seven inorganic cations was prepared: potassium (K), barium (Ba), strontium (Sr), sodium (Na), calcium (Ca), magnesium (Mg) and lithium (Li).

A fused silica capillary externally coated with polyimide was freshly cut from a roll and prepared as described in Example 1. The total capillary length was about 60 cm, and had an internal diameter of 75 μm. The electrolyte was 5 mM 4-methylbenzylamine and 0.021 mM citrate, pH 5.5. The pH was adjusted to pH 5.5 using 2N morpholinoethanesulfonate (MES). A 50 ml beaker and a 100 ml beaker were filled with electrolyte to equal heights. The 50 ml beaker was placed at the anode end of capillary and the 100 ml beaker was placed at the cathode end.

The sample was injected by hydrostatic injection and the separation carried out according to the procedure described in Example 1. The voltage was 25.0 KV. Detection was carried out using a UV/Vis CE detector at a wavelength of 214 nm.

The results are shown in FIG. 8. All seven cations were separated in less than three minutes, and a clear and distinct peak was obtained for each cation. All seven cations were separated within about one minute.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

We claim:

1. A method for detecting cations in a sample using capillary zone electrophoresis comprising the steps of:

a. introducing the sample into a capillary;

b. immersing the capillary into a carrier electrolyte consisting essentially of a UV-absorbing amine or heterocyclic sulfonate compound of similar mobility as the analyzed cation;

c. applying an electrical current under conditions appropriate for the cations in the sample to move along the capillary toward the anode, thereby causing separation of the cations to occur; and

d. detecting the cations indirectly using a UV/visible photometric detector.

2. The method of claim 1 wherein the UV-absorbing amine is 4-methylbenzylamine having a concentration of from about 3 mM to about 10 mM.

3. The method of claim 1 wherein a chelating agent is added to the carrier electrolyte.

4. The method of claim 3 wherein the chelating agent is selected from the group consisting of citrate, succinate, tartrate, hydroxyisobutyrate, and oxalate.

5. The method of claim 1 wherein the electrical voltage is from about 5 to about 40 kV.

6. The method of claim 1 wherein the cations are inorganic cations.

* * * * *

EXHIBIT 9

US005167827A

United States Patent [19]

Glatz

[11] Patent Number: 5,167,827

[45] Date of Patent: Dec. 1, 1992

[54] **CHROMATOGRAPHIC DETERMINATION OF IONS**

[75] Inventor: **Bernd Glatz**, Leonberg, Fed. Rep. of Germany

[73] Assignee: **Hewlett-Packard Company**, Palo Alto, Calif.

[21] Appl. No.: **653,018**

[22] Filed: **Feb. 8, 1991**

[30] **Foreign Application Priority Data**

Apr. 27, 1990 [EP] European Pat. Off. 90108022.6

[51] **Int. Cl.**[5] **B01D 15/08**

[52] **U.S. Cl.** **210/656**; 210/198.2; 422/70; 436/161

[58] **Field of Search** ..... 210/198.2, 656, 635; 73/61.1 C; 422/70; 436/161

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,315,002	4/1967	Small	260/637
4,042,327	8/1977	Haney et al.	210/656
4,265,634	5/1981	Pohl	210/656
4,455,233	6/1984	Pohl et al.	210/656
4,925,567	5/1990	McAleese	210/656
4,927,539	5/1990	Stevens et al.	210/656

OTHER PUBLICATIONS

Frank G. P. Mullins, "Analyst", vol. 112, pp. 665–671 (1987).

B. E. Andrew, "LC–GC", vol. 4, No. 10, pp. 1026–1028 (1987).

E. Heftmann (editor), "Chromatography", vol. 22B, Elsevier Scientific Publishing Co., Amsterdam (1983), pp. B471–B472.

L. R. Snyder and J. J. Kirkland, "Introduction to Modern Liquid Chromatography", Wiley, New York (1979), pp. 292–295, 326–329, 452–483.

Wheals, Journal of Chromatography, vol. 402, pp. 115–126 (1987).

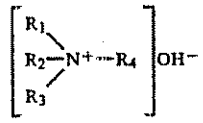
Cassidy and Elchuk, Dynamically Coated Columns for the Separation of Metal Ions and Anions by Ion Chromatography Anal. Chem. vol. 54, pp. 1558–1563 (1982).

Reeve, Journal of Chromatography, vol. 177, pp. 393–397 (1979).

Primary Examiner—Mary Lynn Theisen
Assistant Examiner—Sun Uk Kim

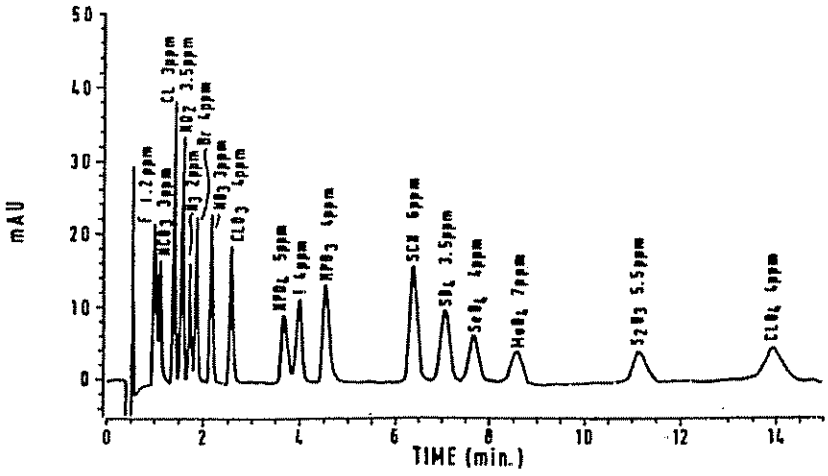
[57] **ABSTRACT**

Herein described is a process for the chromatographic determination of ions, particularly anions on a reversed-phase column, in which sample ions are introduced into a mobile phase containing a modifier and a counterion, the mobile phase with the modifier, counterion and sample ions is passed through the column and, after separation with the aid of a suitable detection method, preferably an indirect UV-detection method, the individual sample ions are determined quantitatively via the decrease the counterion concentration in the eluent. In this process the mobile phase is a mixture of water an an organic solvent and has a pH-value of more than 5. The modifier used is a quaternary ammonium hydroxide of formula



in which at least one radical is a straight-chained or branched alkyl radical with at least 8 and up to 20, more particularly 12 to 18 C-atoms. Prior to the passage of the mobile phase containing the sample ions, the reversed-phase column is preferably brought into equilibrium with the modifier, particularly with the modifier and the counterior. Trimethyl hexadecyl ammonium hydroxide is preferable used as the quaternary ammonium hydroxide. Also described herein is the use of said quaternary ammonium hydroxides for the quantitative chromatographic determination of ions and in particular for the determination of inorganic anions, as well as a reversed-phase material chromatographic column obtained through the conditioning with the modifier.

12 Claims, 7 Drawing Sheets



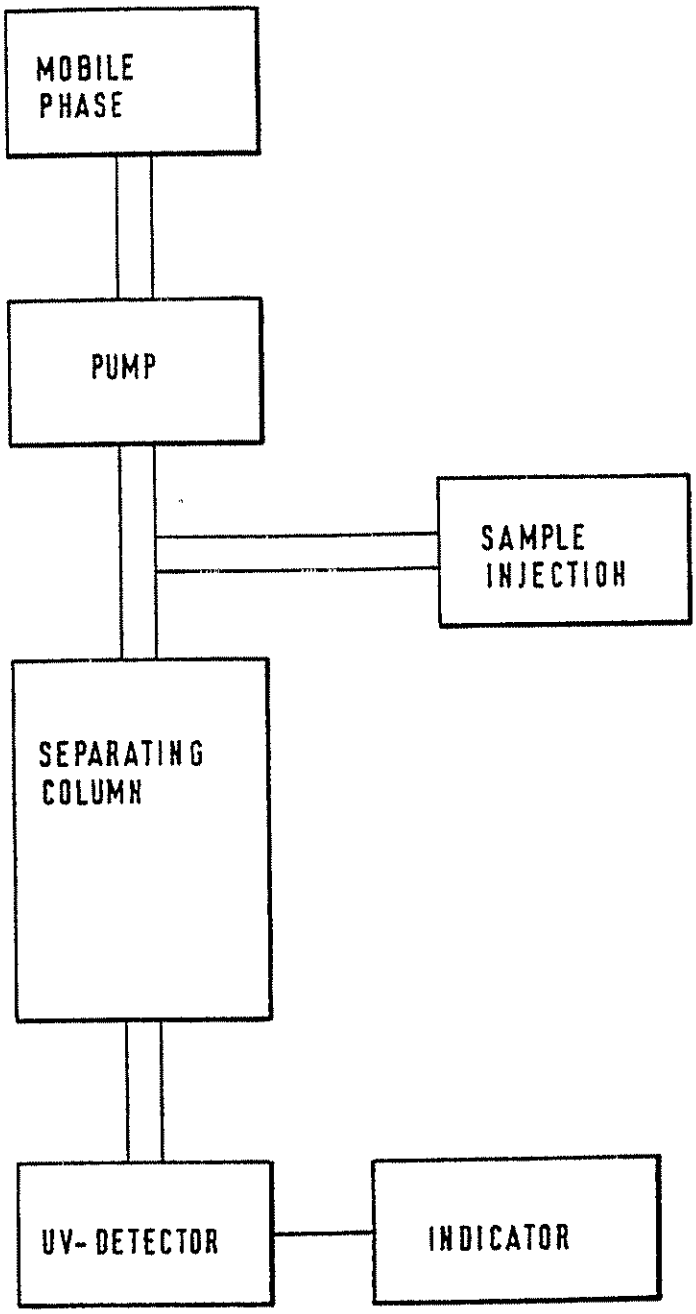

MOBILE PHASE
PUMP
SAMPLE INJECTION
SEPARATING COLUMN
UV-DETECTOR
INDICATOR

FIG. 1

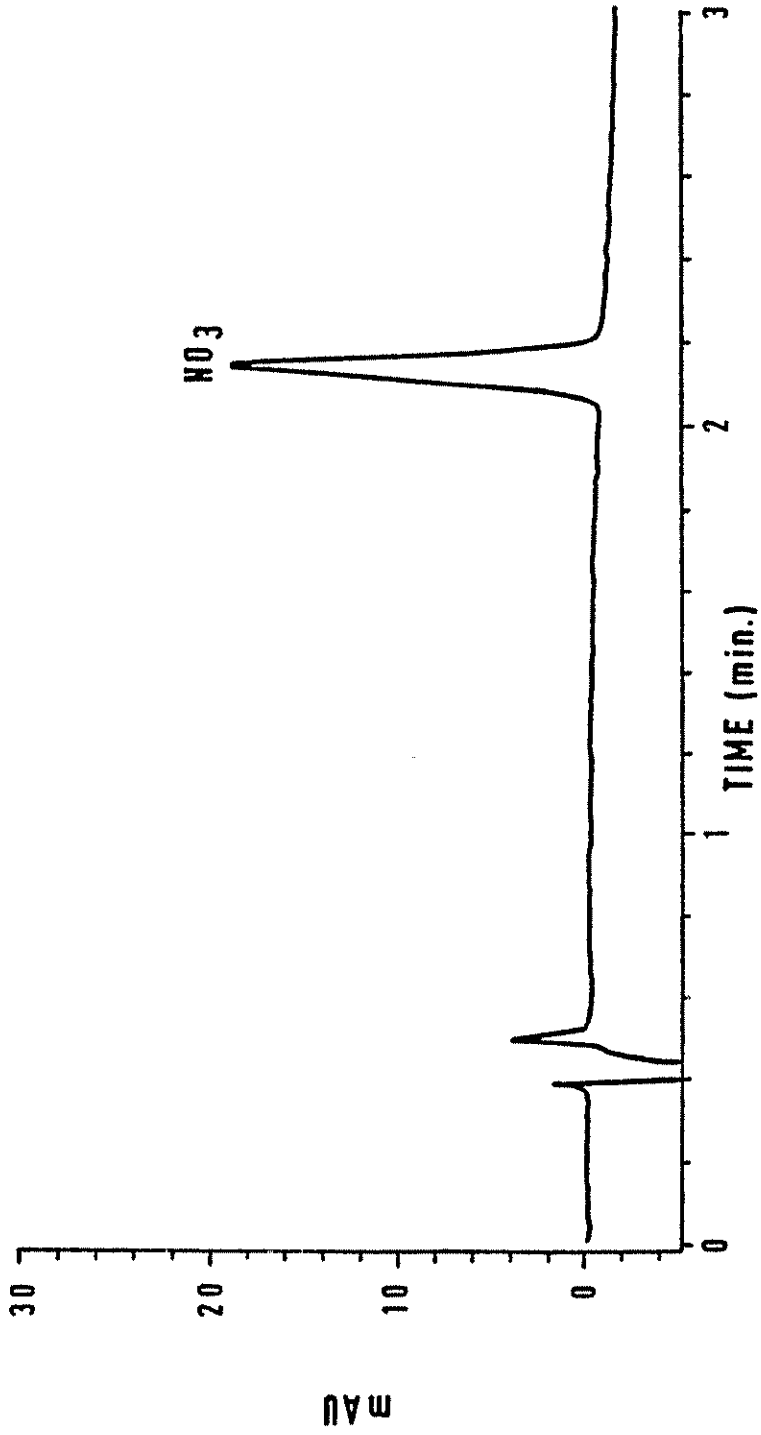


FIG. 2a

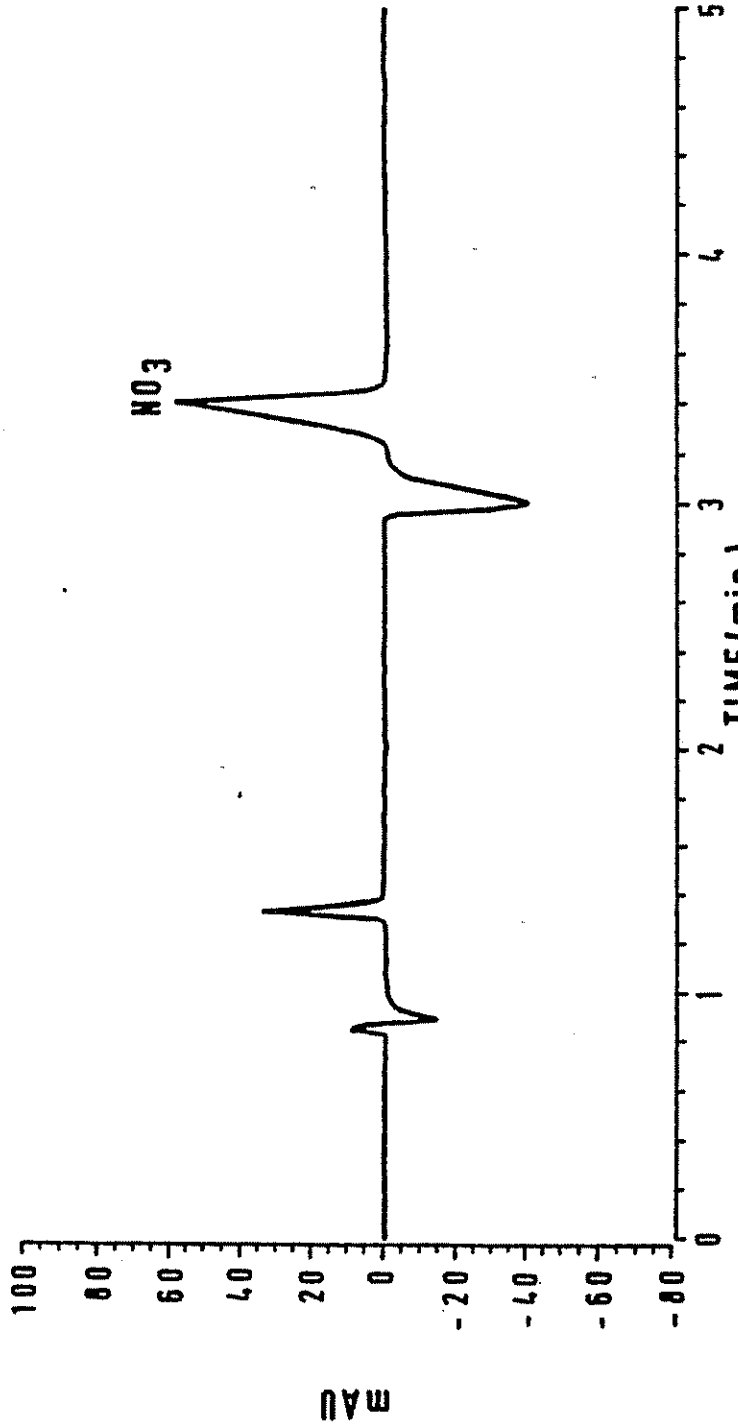


FIG. 2b PRIOR ART

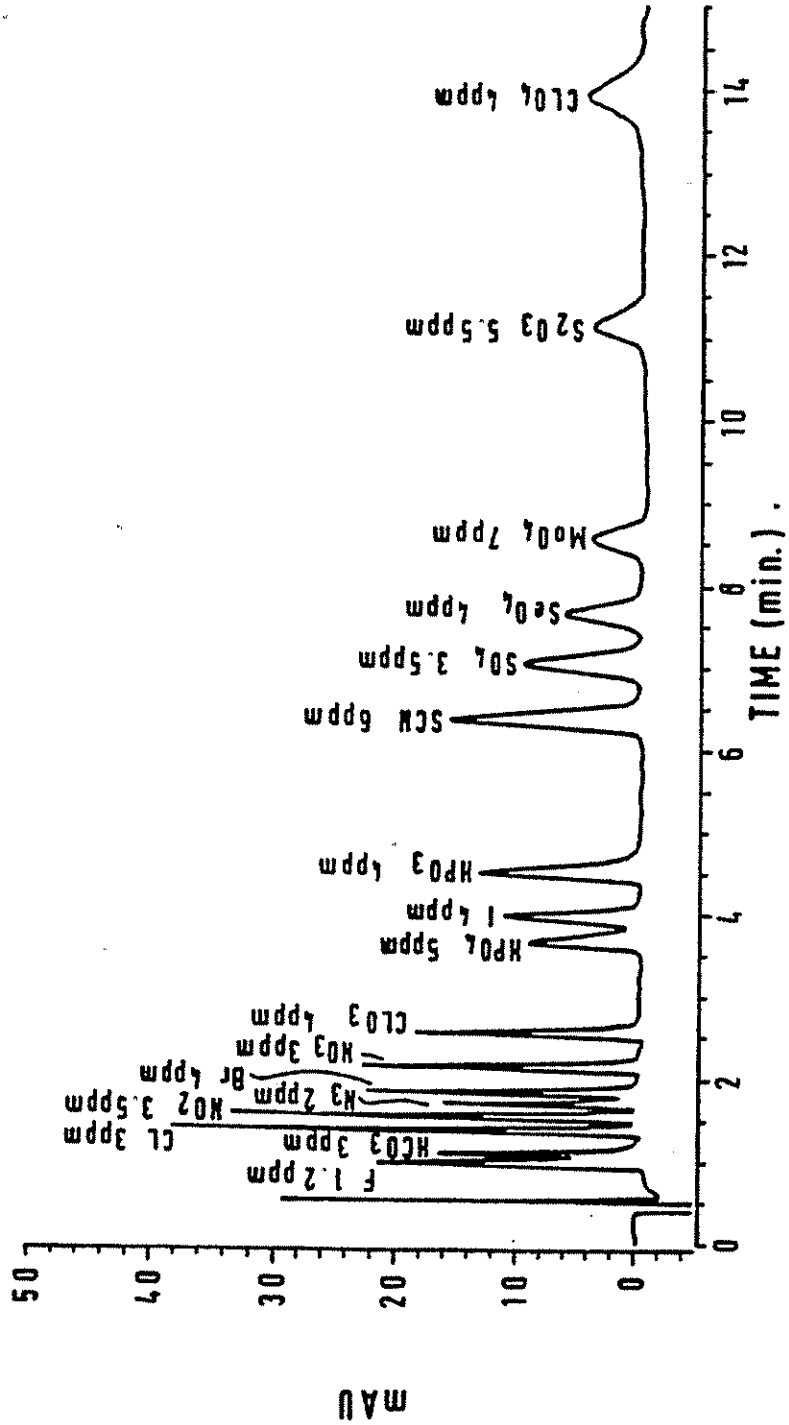

mAU
50
40
30
20
10
0
TIME (min.)
0
2
4
6
8
10
12
14
F 1.2ppm
HCO3 3ppm
CL 3ppm
NO2 3.5ppm
N3 2ppm
Br 1ppm
NO3 3ppm
ClO3 1ppm
HPO4 5ppm
I 1ppm
HPO4 1ppm
SCN 6ppm
SO4 3.5ppm
SeO4 1ppm
MoO4 7ppm
S2O3 5.5ppm
ClO4 1ppm

FIG. 3

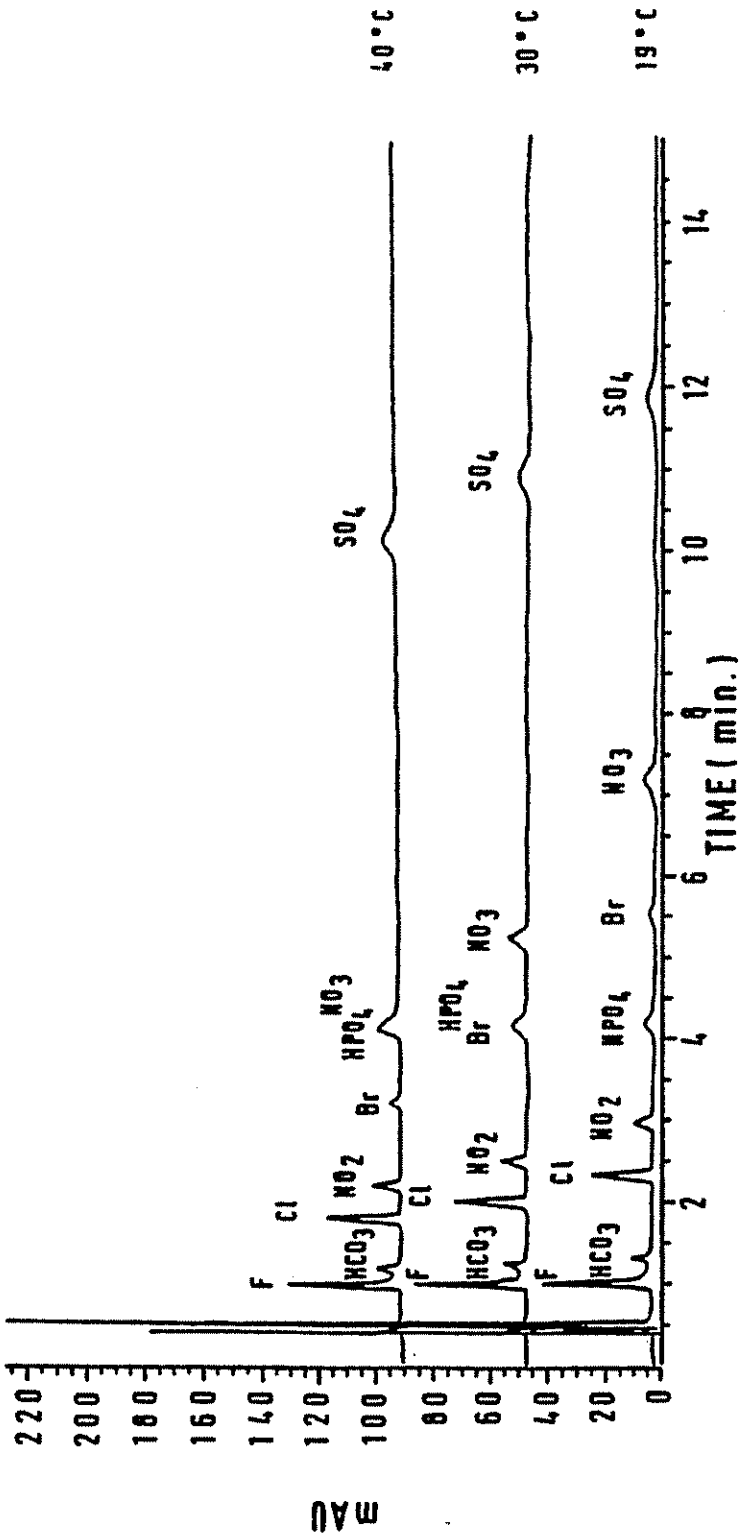

40 °C
30 °C
19 °C
F
Cl
NO2
Br
HPO4
NO3
SO4
HCO3
TIME (min.)
mAU
0
20
40
60
80
100
120
140
160
180
200
220
2
4
6
8
10
12
14

FIG. 4

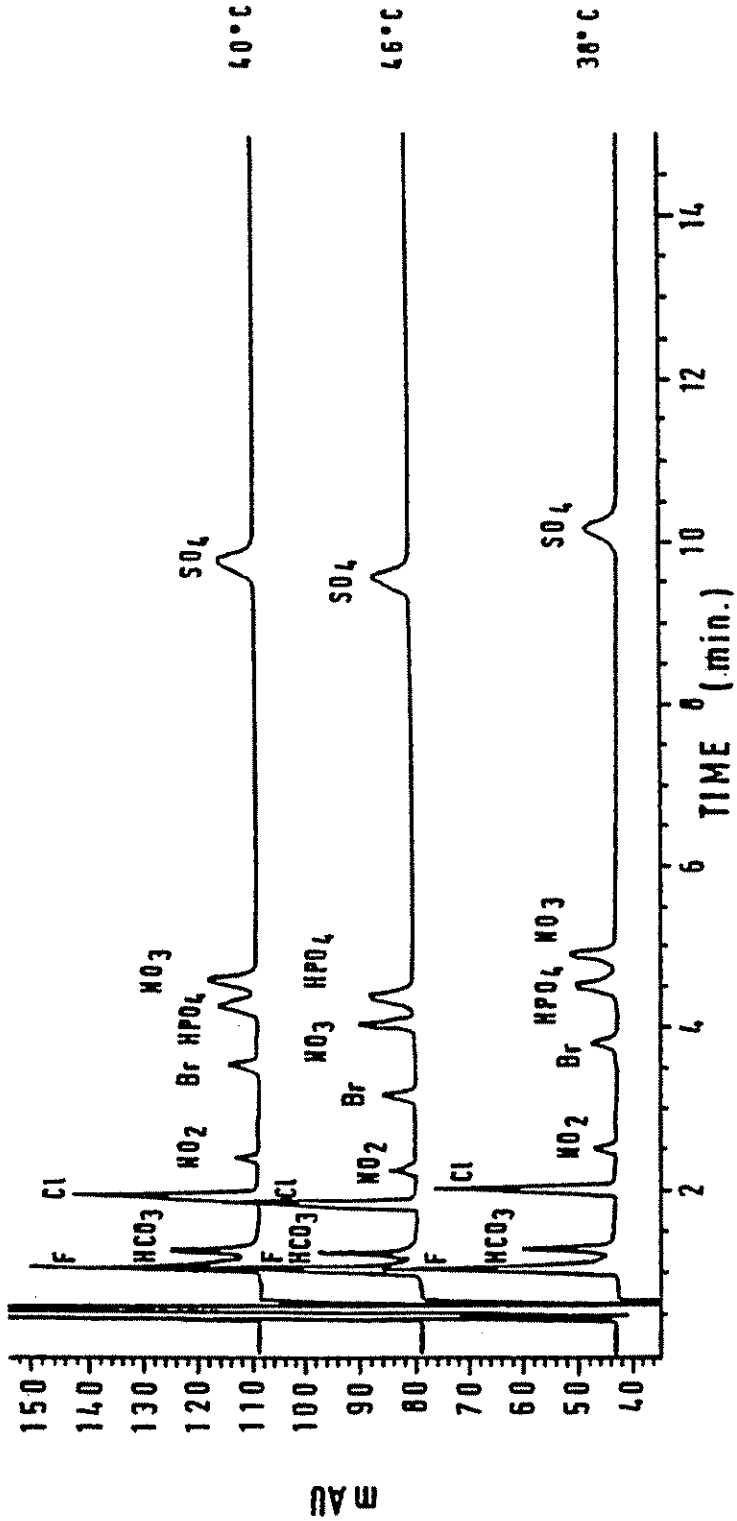

40° C
46° C
38° C
F
Cl
HCO3
NO2
Br
NO3
HPO4
SO4
TIME (min.)
mAU
150
140
130
120
110
100
90
80
70
60
50
40
2
4
6
10
12
14

FIG. 5

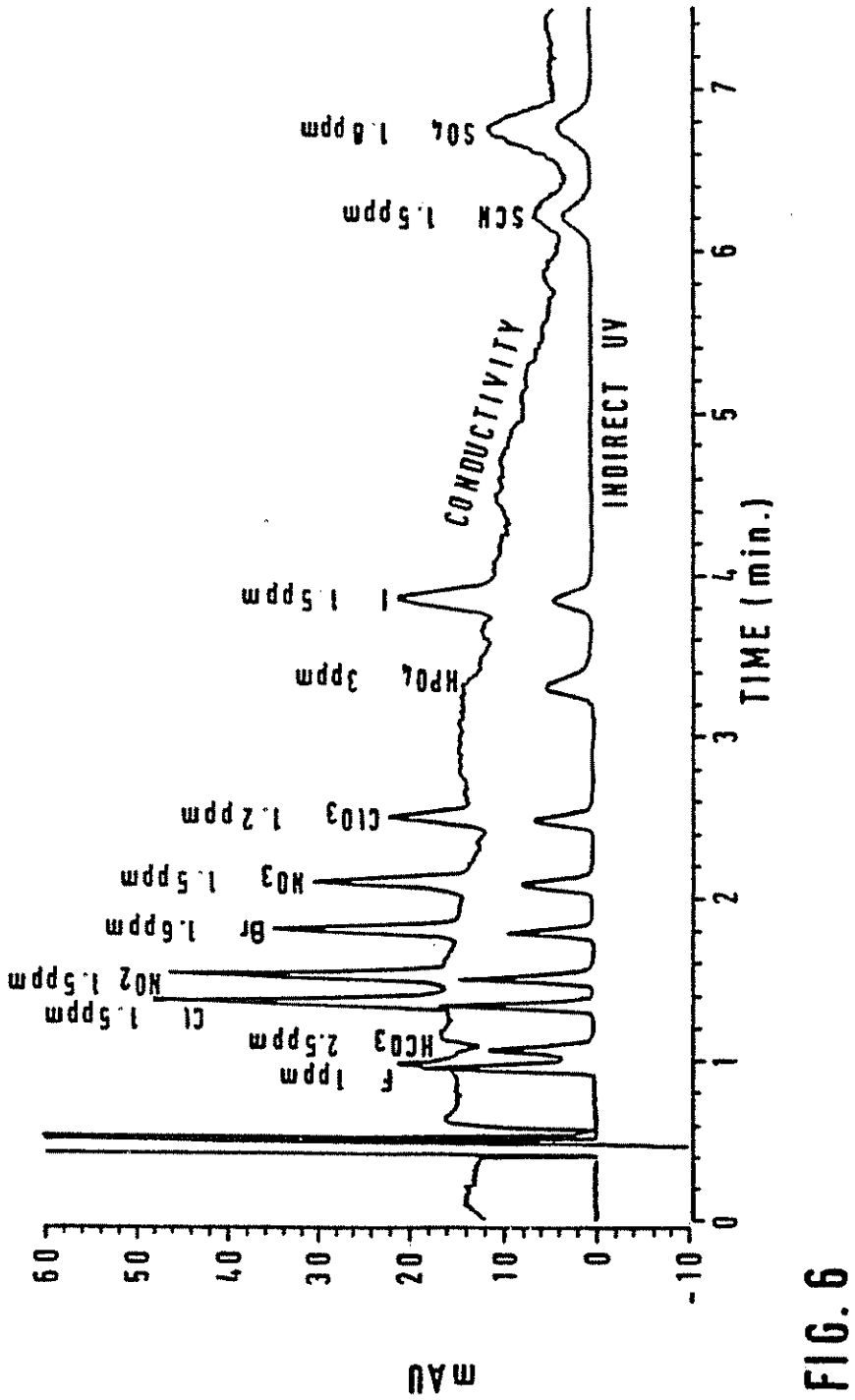

mAU
60
50
40
30
20
10
0
-10
TIME (min.)
0
1
2
3
4
5
6
7
INDIRECT UV
CONDUCTIVITY
F 1ppm
HCO3 2.5ppm
Cl 1.5ppm
NO2 1.5ppm
Br 1.6ppm
NO3 1.5ppm
ClO3 1.2ppm
HPO4 3ppm
I 1.5ppm
SCN 1.5ppm
SO4 1.8ppm

FIG. 6

CHROMATOGRAPHIC DETERMINATION OF IONS

BACKGROUND OF THE INVENTION

The invention relates to a process for the chromatographic determination of ions, particularly anions on a reversed-phase column.

Ever since the arrival of ion chromatography there have been constant improvements to the quantitative analysis of organic and inorganic ions, particularly anions. Due to the poorer column or separation efficiency and the high price, as well as the instability of ion exchange columns, so-called reversed-phase columns have been investigated. In addition, the ions to be determined have been determined by indirect detection methods, such as e.g. indirect identification in the UV-spectral range. However, the chromatograms obtained with reversed-phase columns and indirect identification methods frequently have additional peaks (so-called system peaks) or poorly formed peaks, which make the detection and quantitative determination of the individual ions difficult or even impossible.

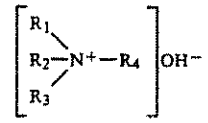
The publication by Frank G. P. Mullins (Analyst, May 1987, Vol. 112, pp. 665 to 671) describes an ion chromatographic process for the determination of inorganic anions by an indirect UV-detection method. The column used is dynamically loaded with hexadecyl trimethyl ammonium bromide. Although the chromatogram has no system peaks, not all inorganic anions can be determined, (as e.g. defined by DIN or EPA standards). Thus, it is not possible to detect fluoride and sulphate ions. In LC-GC, 1987, Vol. 4, No 10, p. 1026 ff, B. E. Andrew describes the use of quaternary ammonium compounds for the ion chromatography of anions. Tetraalkyl ammonium hydroxides are used as ammonium compounds and the straight-chain alkyl radical can contain 1 to 5 C-atoms. In the ion chromatographic determination according to Andrew, system peaks occur in the chromatogram and it is not possible to analyze the anions in one chromatographic run. Andrew also pointed out that a variation in the chain length of the alkyl chains led to no advantages regarding the performance of the process.

SUMMARY OF THE INVENTION

The present invention provides an ion chromatographic column and process which permit a quantitative determination of ions with a high selectivity without disturbing system peaks or peak deformations in the chromatogram. In particular, the invention provides for the chromatographic determination of anions, and preferably inorganic anions, in which the anions can be simultaneously quantified in a single chromatographic run.

This invention is particularly pointed out in the appended claims. In general, the invention provides an ion chromatographic process in which sample ions are introduced into a mobile phase, which contains a modifier and a counterion, the mobile phase with modifier, counterion and sample ions is passed through the column and, following separation the anions are quantified with the aid of a suitable detection method, preferably an indirect detection method, the individual sample ions are quantitatively determined via the concentration decrease of the counterion in the eluent and in which the mobile phase is a mixture of water and an organic solvent and has a pH-value of more than 5 and as the

modifier a quaternary ammonium hydroxide of formula below is used



in which at least one R-radical is a straight-chain or branched alkyl radical with at least 8 and up to 20 and more, particularly 12 to 18 C-atoms. The remaining radicals are the same or different and have 1 to 20 C-atoms, particularly 1 to 10 C-atoms. Preferably the concentration of the ions to be quantitatively determined in the sample is no greater than 500 ppm.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1: a flow chart of an apparatus suitable for performing the inventive process.

FIG. 2*a*: the detection of the nitrate ion according to an embodiment of the inventive process.

FIG. 2*b*: the detection of the nitrate ion according to a prior art comparison process.

FIG. 3: the detection of 17 anions according to an embodiment of the inventive process.

FIG. 4: the temperature dependence of the chromatogram.

FIG. 5: the temperature dependence of the chromatogram according to another embodiment of the inventive process.

FIG. 6: the comparison of the chromatograms of embodiments of the inventive process, in which the ions are detected with the aid of the indirect UV-method and with the aid of the conductivity method.

DESCRIPTION OF THE INVENTION

Compared with known processes, the process according to the invention has the advantages that, by using an inexpensive, stable column and with low overall costs, it is possible to separate in a single chromatographic operation all the ions to be determined and in particular anions and can be quantitatively determined with high sensitivity, without disturbing peaks or peak deformations occurring. Thus, it is possible to establish the presence of and the quantity of fluoride, chloride, bromide, nitrite, nitrate, phosphate and sulphate ions in a single operation, i.e. a single chromatographic run. According to the invention, standard columns and standard HPLC equipment can be used, so that chromatographic determination is possible in a short time with easy handling. Due to the fact that the modifier is used in the hydroxide form, apart from the counterions, no further impurity ions are present, which could disturb the detection of the sample ions.

The invention can be carried out using known reversed-phase columns, particularly those conventionally having carbon chains with 8 to below 20 C-atoms, and in particular reversed-phase columns with C_8, C_{12} and C_{18}-chains which are commercially available. In particular, reversed-phase columns with a C_{18}-chain can be used in the invention. The number of carbon atoms of the reversed-phase column need not coincide with the number of carbon atoms of the modifier, i.e. the alkyl radical of the packing material of the column need not be the same as the alkyl radical of the modifier. According to the invention other standard reversed-phase col-

umn types can be used. These can be columns based on polyacrylamide, which have a C_{18}-chain, or a column based on carbon, whose behavior largely corresponds to a conventional reversed-phase column. Preference is given to the use of columns based on silica. It is possible to use spherical material with a diameter of e.g. 5 μm, but also irregular material.

The length of the column used in the inventive process is more than 2 cm and is normally less than 20 cm, preference being given to a range of 10 to 15 cm. A length below 2 cm is not advantageous, because with such small column length no complete separation of the ions to be determined is possible. A preferred column length is e.g. 12.5 cm.

The flow rate of the mobile phase through the column is not critical. In the case of a 4 mm column diameter, the flow rate is e.g. 0.2 to 5 ml/min, a range of 1 to 3 ml/min being preferred. The detection limit of the inventive process without enrichment is in the lower ppb (parts per billion) range, so that the process is also suitable for the highest demands.

In the case of the inventive process, the mobile phase is preferably allowed to be recycled. Thus, the column can be brought into equilibrium, stable conditions are created and solvent consumption is reduced.

A preferred embodiment of the invention occurs if a quaternary ammonium hydroxide is used, in which the R_1, R_2 and R_3 radicals are the same or different and have 1 to 10 C-atoms and the R_4 radical has between 8 and up to 20, and in particular 12 to 18 C-atoms. Preferably the R_1, R_2 and R_3 radicals are the same and have 1 to 5 C-atoms and the R_4 radical has a straight-chain form and has between 14 and 18, in particular 16 C-atoms. The process performed using said quaternary ammonium hydroxides leads to chromatograms, which permit a particularly good quantitative determination of anions in a single chromatographic run. Particular preference is given to the use in this invention of trimethyl hexadecyl ammonium hydroxide as the quaternary ammonium hydroxide.

The quantity of quaternary ammonium hydroxide modifier contained in the mobile phase can be varied within a wide range and is only dependent on a favorable performance of the process. Preferably the modifier is contained in the mobile phase in a concentration of about 0.05 to 1.5 molar.

An indirect detection method can be used for the detection of the sample ions and the latter can be quantitatively determined via the decrease of the counterion concentration in the eluent. Conductivity, indirect fluorescence and electrochemical measurements are also possible and for the two former methods the increase in the conductivity or the decrease in the fluorescence of the counterion is determined, while in the latter method oxidizable or reducible sample ions are measured. It is also possible, in a modification of the inventive process, to e.g. quantitatively determine anions with the aid of a direct UV-method revealing an absorption in the ultraviolet range of the spectrum. This is preferably the nitrate and nitrite ion. It is particularly advantageous if the indirect detection method is constituted by an indirect determination of the sample ions in the ultraviolet range of the spectrum.

For indirect detection purposes, it is possible to use all counterions which have a detectable physicochemical property and via whose concentration decrease in the eluent the sample ions can be quantitatively determined. Preferably the counterion is the anion of organic acids or sulphonic acids. Such anions are e.g. the anions of a benzene sulphonic, salicylic and in particular phthalic acid. Due to its absorption in the ultraviolet range of the spectrum, the phthalate ion is particularly suitable for performing the inventive process, in conjunction with the indirect determination of the sample ions in the UV-spectral range. The counterion concentration in the mobile phase can be varied over a wide range. A too high concentration increase leads to a sensitivity loss and to a poorer separation of the individual ions. If the concentration is excessively reduced, the column capacity may be low. A preferred concentration range for the counterion in the mobile phase is between about 6×10^{-3} to 1×10^{-4} and in particular about 6×10^{-3} to 4×10^{-4} molar.

The mobile phase containing the quaternary ammonium hydroxide modifier and the counterion comprises a mixture of water and an organic solvent. It is possible to use various organic solvents, such as e.g. acetonitrile, methanol, dimethyl formamide and dimethyl sulphoxide. The use of less polar organic solvents is possible, the first-mentioned polar, organic solvents and more especially acetonitrile being preferred. Particularly good results are obtained when using a mobile phase comprising a mixture of about 65 to 95 vol % (and in particular approximately 80 vol %) of water and about 5 to 35 vol % (and in particular approximately 20 vol %) of organic solvent.

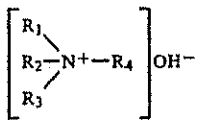
In the process according to the invention, the mobile phase has a pH-value of more than 5. pH-values between 6 and 12 and more especially between about 7 and 9 are preferred. If the pH-value is in the alkaline range, it can be adjusted with the aid of a base, particularly sodium hydroxide solution. Through the choice of the pH-value, it is possible to control the inventive process in such a way that under all circumstances the simultaneous detection of all the ions to be determined in a single chromatographic operation is possible.

The process according to the invention can be performed at elevated temperature, at ambient temperature and also at lower than ambient temperatures. Preferably the temperature is between about 0° and 80° C., particularly between about 20° and 50° C. Thermostatic control can e.g. take place at higher than ambient temperatures by placing the column in a thermostatically controlled area (oven). It is advantageous if the chosen temperature is kept substantially constant during the process, preferably with a variation of max. ±1° C. This temperature constancy may be necessary if the chromatogram has a different appearance at different temperatures. Thus, it is possible that the time intervals with which the anions are eluted by the column will vary with the temperature, or for there even to be a peak sequence reversal. This phenomenon can be attributed to the temperature dependence of the phase equilibria between the stationary and mobile phase. Preferably the temperature is kept constant with a variation of max. ±0.5° C. and in particular ±0.2° C. If e.g. the precision with regards to the reproducibility of the times at which the individual ions are eluted by the column is to be better than 1%, then the set temperature kept constant with a temperature fluctuation of max. ±0.5° C. If several chromatographic operations are performed successively, the temperature at which the process is performed is not only kept constant during the individual analyses, but also between said analyses, so that reproducible and comparable results are obtained. The embodiments of the inventive process, in

which the temperature is kept constant, are particularly suitable for use in an automated and in particular a computer-controlled operation, evaluated with the aid of a computer.

The hydraulic pressure under which the mobile phase is passed through the column is dependent on the packing material and can be varied within wide limits If spherical packing materials are used as the column material, a hydraulic pressure of 50 to 200 bar is preferred at a particle diameter of e.g. 5 μm. If smaller diameter particles, e.g. 3 μm are used, it can be appropriate to use a higher pressure, the pressure rising quadratically with decreasing particle diameter.

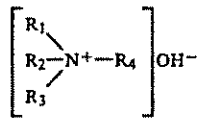
The invention also relates to the use of a quaternary ammonium hydroxide of formula



for the quantitative chromatographic determination of ions, in which at least one of the R-substituents is a straight-chain or branched alkyl radical with at least 8 and up to 20, more particularly 12 to 18 C-atoms and the remaining radicals are the same or different and have 1 to 20 and in particular 1 to 10 C-atoms. It is advantageous if a quaternary ammonium hydroxide is used, in which the R_1, R_2 and R_3 radicals are the same and have 1 to 5 C-atoms and in which the R_4 radical has a straight-chain form and has between 4 and 18, particularly 16 C-atoms. Once again the use of trimethyl hexadecyl ammonium hydroxide is preferred among these quaternary ammonium hydroxides.

The inventive use inter alia relates to the quantitative determination of ions, particularly anions, in water, such as e.g. in waste or drinking water, in food stuffs, such as e.g. beer, wine, juices or vegetables, as well as in biotechnological and physiological substances, such as blood and urine, or in liquids used in the electrical more particularly to the use of quaternary ammonium hydroxides for determining inorganic ions in a single chromatographic operation and preferably at least fluoride, chloride, bromide, nitrite, nitrate, phosphate and sulphate are simultaneously quantified.

Finally, the invention relates to a chromatographic column, particularly suitable for performing the inventive process, in which the column contains a reversed-phase material, which is conditioned with a modifier, more particularly a modifier and a counterion and the modifier is a quaternary ammonium hydroxide of formula



in which at least one of the R-substituents is a straight-chain or branched alkyl radical with at least 8 and up to 20, more particularly 12 to 18 C-atoms and the remaining radical are the same or different and have 1 to 20, particularly 1 to 10 C-atoms. The reversed-phase material of the chromatographic column is preferably conditioned with a modifier, whose R_1, R_2 and R_3 radicals are the same and have 1 to 4 C-atoms and whose R_4 radical has a straight-chain form and has between 14 and 18 and in particular 16 C-atoms. Preferably the quaternary ammonium hydroxide is used for conditioning purposes is trimethyl hexadecyl ammonium hydroxide. The inventive column leads to a very good separation of all ions and in particular anions to be determined in a single operation and with high sensitivity. The column can be obtained at low costs and the column selectivity is maintained even after a large number of determinations, such as e.g. after more than 1000 injections.

The following examples and drawings serve to illustrate the invention. In the examples and drawings the individual features can be realized either singly, or in the form of random combinations.

FIG. 1 is the flow chart of an apparatus for performing an embodiment of the process according to the invention. By means of a pump, the mobile phase is pumped through the chromatographic column and the counterions contained in the mobile phase are visible by a UV-detector. There is also a sample injection enabling the sample ions to be introduced into the mobile phase. The inventive process is normally performed as follows. A mobile phase of a mixture of water and an organic solvent is prepared, which contains a modifier and a counterion in a concentration of 0.05 to 1.5 molar or 6×10^{-3} to 1×10^{-4} molar and which has a pH-value of more than 5. This mobile phase is pumped through the chromatographic column using a liquid pressure pump at a pressure of about 50 to 200 bar, the column being kept at a temperature between 20° and 80° C. with a variation of max. ±1° C. approximately 2 to 4 hours an equilibrium has established between the stationary and mobile phase. By means of sample injection, the sample ions to be determined are then applied to the chromatographic column and are successively eluted by the latter, accompanied by a further continuous passage of the mobile phase. The individual ions are quantitatively determined via the concentration decrease of the counterion in the eluent.

EXAMPLE 1

A 200 mm long and diameter 4.6 mm chromatographic column is filled with Hypersil ODS, of 5 μm spherical particles. This column is conditioned with a mobile phase of 73% water and 27% acetonitrile, which contains 0.8 mmole of a hexadecyl trimethyl ammonium ion and 2 mmole of phthalic acid and is set to a pH-value of 7.2 with a sodium hydroxide solution. The mobile phase flow rate is 2 ml/min. The phthalate counterion is detected at 254 nm. After the equilibrium has been established between the stationary and mobile phase a sample solution, which contains the nitrate ions, is introduced into the mobile phase and the elution of the nitrate ions by the column is observed.

FIG. 2*a* shows the chromatogram when using hexadecyl trimethyl ammonium hydroxide as the modifier Roughly 2 minutes after the application of the sample solution, a symmetrical peak appears from which the content of nitrate ions can be quantitatively determined.

FIG. 2*b* shows the chromatogram when hexadecyl trimethyl ammonium bromide is used as the modifier. After roughly 3 minutes a disturbing system peak appears, which does not occur when performing the inventive process.

EXAMPLE 2

The column described in Example 1 is conditioned with the mobile phase containing hexadecyl trimethyl ammonium hydroxide as the modifier. The other conditions are as in Example 1. After establishing the equilibrium, a sample solution containing 17 anions is introduced into the mobile phase. The chromatogram shown in FIG. 3 is obtained within 15 minutes. Thus, in a single chromatographic operation all 17 anions are separated from one another and can be quantitatively determined.

EXAMPLE 3

A 100 mm long, diameter 4.6 mm column is filled with Hypersil ODS. This column is then conditioned with a mobile phase of 82% water and 18% acetonitrile for 3 hours for obtaining an equilibrium. The mobile phase contained 0.8 mmole of hexadecyl trimethyl ammonium hydroxide and 2 mmole of phthalic acid and is set to a pH-value of 8.4 with a sodium hydroxide solution. The flow rate of the mobile phase is 2 ml/min. The phthalic counterions are detected at a wavelength of 266 nm. In three different tests, the chromatographic column is thermostated to 3 different temperatures, namely 19° C., 30° C. and 40° C., the temperature being kept constant with an accuracy of ±0.5° C. or less. After setting the equilibrium, 50 µl of sample solution containing 8 different anions are injected. These anions are eluted by the column through the mobile phase and determined with the aid of the indirect UV-method.

FIG. 4 shows the results of 3 chromatographic determinations at the 3 different temperatures. At 19° C. there is a complete separation of all the anions, so that a separate quantitative determination of the anions is possible. At 30° C. the bromide and phosphate ions are not completely separated from one another, but the other six anions can be quantified. At 40° C. the nitrate and phosphate ions are not completely separated from one another, but the other six anions can be quantified.

FIG. 4 shows that through the choice of suitable conditions and in particular the temperature setting, it is possible to achieve a simultaneous determination of all the anions in a single chromatographic step.

EXAMPLE 4

A 125 mm long, diameter 4.0 mm chromatographic column is filled with Lichrospher 100 RP-18 and is conditioned with a mobile phase of 82% water and 18% acetonitrile, which contains 0.8 mmole of hexadecyl trimethyl ammonium hydroxide and 2 mmole of phthalic acid and is adjusted to a pH-value of 8.6 with a sodium hydroxide solution. The flow rate is 2.0 ml/min. Conditioning takes place for approximately 2 to 4 hours and is performed in three different passages at 38° C., 40° C. and 46° C. The temperature is kept constant with an accuracy of ±1° C. by a suitable thermostat (oven). After setting the equilibrium, 50 µl of a sample solution containing 8 different anions is introduced into the mobile phase. The counterions are detected at a wavelength of 266 nm.

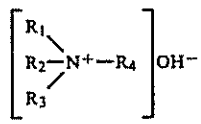
FIG. 5 shows the results of the determinations performed at said 3 temperatures. It can be seen that all 3 temperatures there is a complete separation of the individual anions and a quantitative determination of the individual ions is possible.

EXAMPLE 5

A column filled with Hypersil ODS according to Example 2 is conditioned as in the latter. A sample solution containing 11 different anions is then introduced into the mobile phase. The individual ions are eluted by the column and detected once with the indirect UV-method and on a further occasion with the conductivity method. The results of these two determinations are given in FIG. 6. The latter shows that both with the conductivity method and with the indirect UV-method, a determination of the anions contained in the sample solution is possible in one chromatographic run.

I claim:

1. A process for the chromatographic determination of ions without disturbing system peaks or peak deformations in the chromatogram in a reversed-phase chromatographic column which process comprises introducing sample ions into a mobile phase containing a modifier and a counterion, passing the mobile phase with the modifier, counterion and sample ions through said column, separating said sample ions and detecting the quantitatively determining the individual sample ions with the aid of an indirect ultraviolet detection method via the decrease in the concentration of the counterion in the eluent, in which process the mobile phase is a mixture of water and an organic solvent having a pH-value of between about 7 and 9, and the modifier is a quaternary ammonium hydroxide of formula



in which the R_1, R_2, R_3 and R_4 radicals are alkyl chains, which are the same or different and have 1 to 20 C-atoms and in which at least one radical is a straight-chain or branched alkyl radical with at least 8 and up to 20 C-atoms.

wherein the mobile phase contains about 5–35 volume percent polar organic solvent in water,

wherein said modifier is present in the mobile phase in a concentration from about 0.05×10^{-3} to 1.5×10^{-3} molar, and said counterion is the anion of phthalic acid present in the mobile phase in a concentration from about 6×10^{-3} to 1×10^{-4} molar.

2. Process according to claim 1, wherein said column is a reversed-phase column and prior to said step of passing through of the mobile phase containing the sample ions, the column is brought into equilibrium with the modifier and the counterion.

3. Process according to claim 2 wherein said column is brought into equilibrium with a mobile phase which, apart from the sample ion content, has the same composition as the mobile phase used for determining the sample ions.

4. Process according to claim 1 wherein R_1, R_2 and R_3 are the same or different and have 1 to 10 C-atoms and R_4 has between 12 and 18 C-atoms.

5. Process according to claim 4 wherein R_1, R_2 and R_3 are the same and have 1 to 5 C-atoms and R^4 has a straight-chain form and has between 14 and 18 C-atoms.

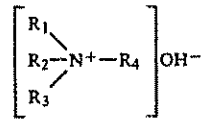
6. Process according to claim 1 wherein trimethyl hexadecyl ammonium hydroxide is the quaternary ammonium hydroxide.

7. Process according to claim 1 wherein the counterion is contained in the mobile phase in a concentration of about 6×10^{-3} to 4×10^{-4} molar.

8. Process according to claim 1 wherein said process is performed at a temperature between about 0° and 80° C.

9. Process according to claim 1 wherein the temperature is kept constant during the process, with a maximum variation of ±0.5° C.

10. A process for the chromatographic determination of anions without disturbing system peaks or peak deformations in the chromatogram in a reversed-phase chromatographic column, which process comprises: conditioning said column to equilibrium with a mobile phase containing a modifier and a counterion with mobile phase contains about 5-35 vol. % polar organic solvent in water, introducing sample ions into said mobile phase and passing said mobile phase through said column, and separating said sample ions and detecting and quantitatively determining with the aid of an indirect ultraviolet detection method the individual sample ions via the decrease in the concentration of the counterion in the eluent, in which process the mobile phase has a pH between about 7 and 9, and the modifier is a quaternary ammonium hydroxide of the formula



wherein R_1, R_2 and R^3 are alkyl radicals having from 1 to 5 c-atoms and R_4 is a straight chain alkyl radical having from about 14 to 18 C-atoms, said modifier being present in the mobile phase in a concentration from about 0.05×10^{-3} to 1.5×10^{-3} molar, and said counterion being a phthalate ion present in said mobile phase in a concentration from about 6×10^{-3} to 4×10^{-4} molar.

11. Process according to claim 10 wherein the quaternary ammonium hydroxide is trimethyl hexadecyl ammonium hydroxide.

12. Process according to claim 10 wherein said determination of ions is for determining fluoride, chloride, bromide, nitrite, nitrate, phosphate and sulphate in a single chromatographic run.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,167,827

DATED : December 1, 1992

INVENTOR(S) : Glatz

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In column 5, line 41, after "electrical" insert --industry such as e.g., plating baths. The invention relates--.

In column 6, line 27, delete "concentration" and substitute --concentrate--.

In column 6, line 27, delete "molar" and substitute --mmole--.

In column 6, line 28, delete "molar" and substitute --mmole--.

In column 6, line 33, after "C." insert --by means of a suitable thermo-stating device. After--.

Signed and Sealed this

Twenty-eight Day of March, 1995

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

EXHIBIT 10

US005240576A

United States Patent [19]

Lauer et al.

[11] Patent Number: 5,240,576

[45] Date of Patent: Aug. 31, 1993

[54] CAPILLARY ELECTROPHORESIS

[75] Inventors: Henk H. Lauer, Belmont; Paul D. Grossman, Redwood City; Dennis E. Mead, Campbell, all of Calif.

[73] Assignee: Applied Biosystems, Inc., Foster City, Calif.

[21] Appl. No.: 461,568

[22] Filed: Jan. 5, 1990

Related U.S. Application Data

[62] Division of Ser. No. 156,430, Feb. 16, 1988, abandoned.

[51] Int. Cl.[5] G01N 27/26; B01D 57/02

[52] U.S. Cl. ..... 204/180.1; 204/299 R

[58] Field of Search ..... 204/299 R, 180.1, 183.2

[56] References Cited

U.S. PATENT DOCUMENTS

3,664,939	5/1972	Luner et al.	204/183.2
4,898,658	2/1990	Karger et al.	204/180.1 X
4,906,344	3/1990	Hjerten	204/180.1 X

OTHER PUBLICATIONS

"High-Resolution Separations Based on Electrophoresis and Electroosmosis", Jorgenson, J. W., Lukacs, K. D., *J. of Chromatography*, 218 (1981) pp. 209–216.

"Capillary zone Electrophoresis", Jorgenson, J. W.,k Lukacs, K. D., *Science*, vol. 222, 21 Oct. 1983.

"Characterization of A Microinjector for Capillary Zone Electrophoresis", Wallingford, R. A., Ewing, A. G., *Anal. Chem.*, 1987, 59, 681–684.

"Separation of Organic and Metal Ions by High-Voltage Capillary Electrophoresis", Tsuda, T., Nomura, K., Nakagawa, G., *J. of Chromatography*, 264 (1983) pp. 385–392.

"Evaluation of an Automatic Siphonic Sampler for Capillary Zone Electrophoresis", Honda, S., Iwase, S., Fujiwara, S., *J. of Chromatography*, 404 (1987) pp. 313–320.

"Electric Sample Splitter for Capillary Zone Electrophoresis", Deml, M., Foret, F. Bocek, P., *J. of Chromatography*, 320 (1985) pp. 159–165.

"Theory, Instrumentation, and Applications of Capillary Zone Electrophoresis", Lukacs, K. D., Dissertation to University of North Carolina at Chapel Hill, 1983.

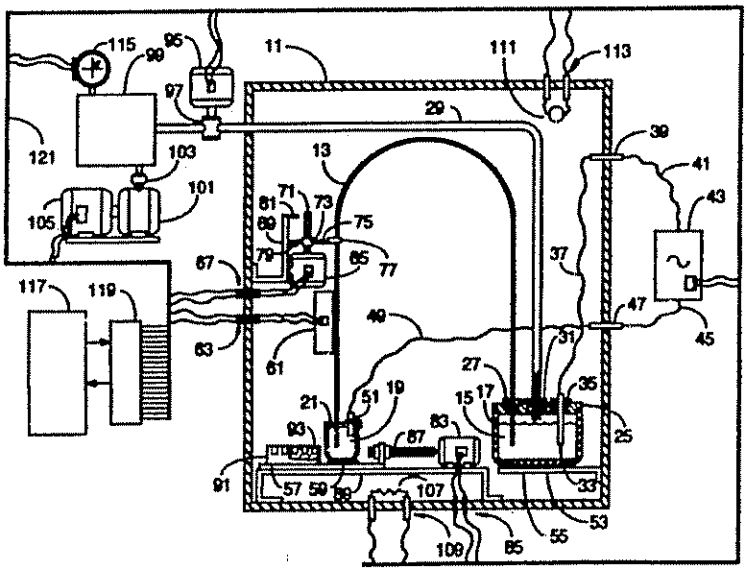
"Automatic Liquid Chromatograph Injection and Sampling", Kretz, Wolfgang, and Hans-Georg Hartl, *Hewlett Packard Journal*, pp. 21–24, Apr. 1984.

Primary Examiner—John F. Niebling
Assistant Examiner—John S. Starsiak, Jr
Attorney, Agent, or Firm—Joseph H. Smith

[57] ABSTRACT

an apparatus is disclosed for providing capillary electrophoresis, which includes an electronically controlled valve system for automatically introducing a sample into the capillary by means of a vacuum at the end of the capillary tube. This approach of sucking in the sample is extremely accurate and reproducible, and results in a minimum of band broadening. Furthermore, it enables the entire capillary electrophoresis sytem to be easily automated. An automated temperature control system is provided which enables the temperature of the capillary tube (and hence the solvent/solute system) to be controlled during electrophoresis, thereby very directly controlling pH and electrophoretic mobility. In another embodiment, the capillary is prewashed and equilibrated to achieve substantially zero charge on the capillary wall, thereby essentially eliminating electroosmotic flow and substantially improving resolution.

6 Claims, 3 Drawing Sheets



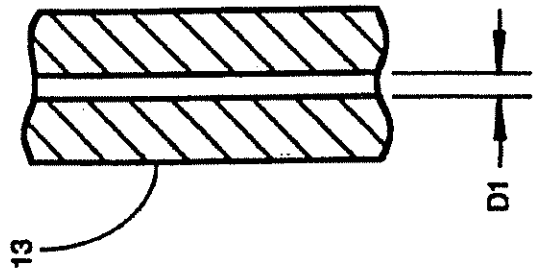

13
D1

Fig. 1B

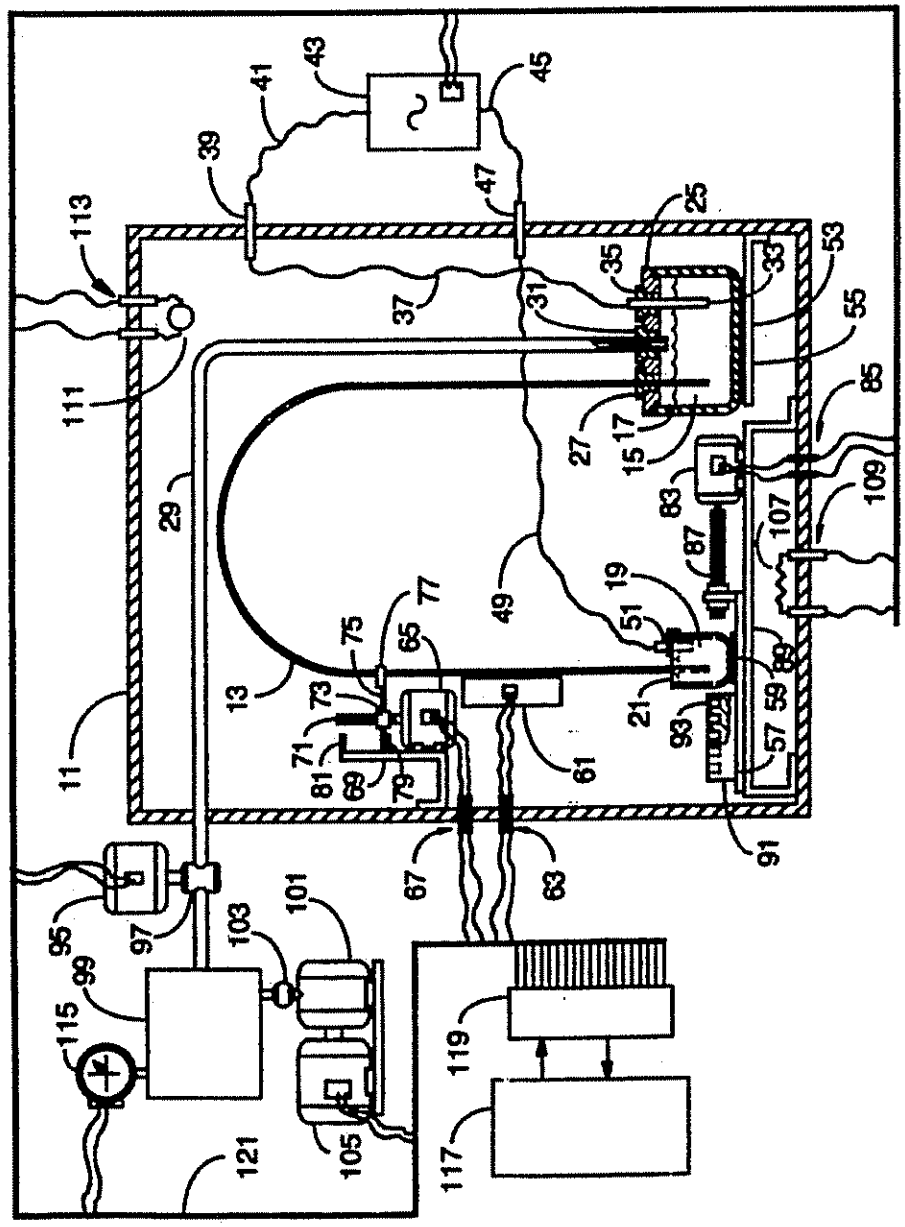

11
13
15
17
19
21
25
27
29
31
33
35
37
39
41
43
45
47
49
51
53
55
57
59
61
63
65
67
69
71
73
75
77
79
81
83
85
87
89
91
93
95
97
99
101
103
105
107
109
111
113
115
117
119
121

Fig. 1A

Run Number	Buffer	pH Level	Elution time Electroosmotic Flow (min)	Pre-Wash	Electroosmotic Mobility 10^{-4} cm^2/Vs
#1	Caps (10 nM)	pH = 11.13	t = 1.70	NaOH	8.2
		pH = 11.13	t = 1.63	HCl	8.5
#2	Bicene (10 nM)	pH = 8.45	t = 1.64	NaOH	8.5
		pH = 8.45	t = 1.93	HCl	7.2
#3	MES (10 nM)	pH = 6.0	t = 2.09	NaOH	6.7
		pH = 6.0	t = 6.27	HCl	2.2
#4	MES (10 nM)	pH = 6.0	t = 2.07	NaOH	6.7
		pH = 6.0	t = 4.80	HCl	2.9
#5	Citric Acid (20 nM)	pH = 4.0	t = 11.82	NaOH	1.2
		pH = 4.0	t = 54.6	HCl	0.25
#6	Citric Acid (20 nM)	pH = 2.51	t = 36.53	NaOH	0.38
		pH = 2.51	t = 70.31	HCl	0.2

Fig. 2

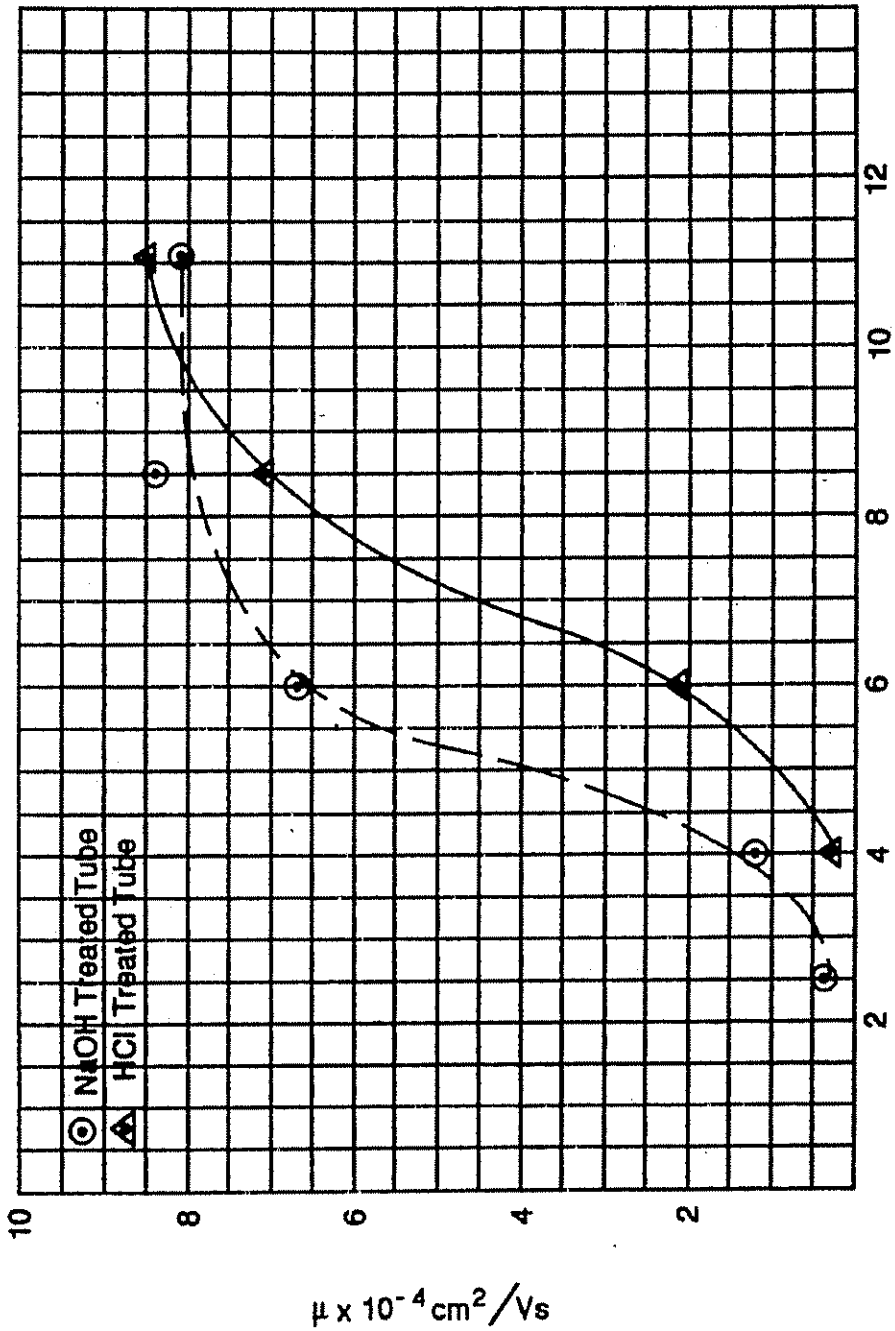

NaOH Treated Tube
HCl Treated Tube
pH
12
10
8
6
4
2
10
8
6
4
2
μ x 10⁻⁴ cm²/Vs

Fig. 3

CAPILLARY ELECTROPHORESIS

This is a continuation, of application Ser. No. 156,430, filed Feb. 16, 1988, now abandoned.

BACKGROUND OF THE INVENTION

This invention relates to capillary electrophoresis, or as it is more conventionally called "capillary zone electrophoresis" (CZE), and more particularly to automated methods and apparatus for introducing samples into capillary columns and for improving separations by using temperature control of said columns.

In recent years significant advances have been made in micro-column separation techniques. A principal advantage of such techniques is their suitability for analysis of extremely small sample volumes, e.g. in the microliter or submicroliter amounts of sample. Beino able to analyze such small volumes has become exceedingly important with the explosion of research in the biological field, because often-times biological samples are quite small.

One of the significant problems with capillary techniques is in introducing sample into the capillary. One technique used in capillary electrophoresis, called sample injection, is electromigration, a term collectively including the effects of eletrophoresis and electro-osmosis (See Jorgenson, J. W, and Lukacs, K. D., *J. Chromatography,* 1981, Vol. 218, pp. 209–216; Jorgenson, J. W., and Lukacs. K. D., *Science,* 1983, Vol. 222, pp. 266–272; and Wallingford, R. A. and Ewing, A. G., *Anal Chem.,* 1987, Vol. 59, pp. 681–684). In this technique, one end of the capillary and the electrophoresis anode are placed into the sample and a voltage is briefly applied, causing a small band of sample to electromigrate into the capillary. This method of sample injection suffers from discrimination within the sample because solutes with higher mobilities will preferentially migrate into the electrophoresis column and therefore change the relative composition of the sample. To avoid this problem, attempts to physically inject sample have also been reported (Jorgenson and Lukacs, *Science,* ibid). However these direct injection techniques cause band broadening, apparently due to the laminar flow profile introduced during the injection.

Other less common injection methods include gravity flow (See Tsuda, A., et al, *J. Chromatography,* 1983, Vol. 264, pp. 385–392.), siphoning (See Honda, S. et al, *J. Chromatography,* 1987, Vol. 404, pp. 313–320.), and the use of an electonic sample splitter (See Deml, M. et al, *J. Chromatography,* 1985, Vol. 320, pp. 159–165.). Each of these injection techniques are capable of placing subnanoliter volumes of sample into the electrophoresis column with a minimum of band broadening. However, the gravity flow or siphoning injection method is inaccurate and lacks precision in providing absolute volume amounts due to the unreliable position of the sample level which will change due to the sample withdrawal. The latter can only be neglected if the original sample volume is large compared to the volume injected. With the electronic splitter, a larger initial sample volume is required in order be able to split it down to the smaller size required for the column. Thus, some sample may be wasted, or there may not be sufficient sample to perform a separation. Also, this latter technique is further complicated by requiring an additional controlled power supply or very careful control of the electric resistances in the different legs of the splitter. Furthermore, the need to use an initial larger sample size significantly decreases the number of applications for which it can be used.

What is needed is a simple, automatable, sample injection technique that is suitable for microvolumes, is capable of providing accurate sample volumes, and which produces a minimum of band broadening.

SUMMARY OF THE INVENTION

In accordance with preferred embodiments of the invention, an apparatus is disclosed for providing capillary electrophoresis, which includes an electronically controlled valve system for automatically introducing a sample into the capillary by means of a vacuum at the end of the capillary tube. This approach of sucking in the sample is extremely accurate and reproducible, and results in a minimum of band broadening. Furthermore, it enables the entire capillary electrophoresis sytem to be easily automated.

The apparatus includes first and second reservoirs that are electrically isolated from each other for holding electrophoretic media, a sample reservoir located in proximity of the first reservoir for holding a sample to be electrophoresed, and a high voltage power supply connected between the first reservoir and the second reservoir. A first pressure source of a first gas having a first known pressure, typically the ambient air pressure, is used for providing an environment for the first reservoir and the sample reservoir, so that electrophoretic media in the first reservoir and sample in the sample reservoir are under the first pressure. The apparatus includes a pressure reservoir for holding a second gas (also typically air) having a second pressure that is lower than the first pressure A capillary tube is also included in which to electrophorese the sample. A rack system is provided for holding the first and second reservoirs, the pressure reservoir, the high voltage power supply, the sample reservoir, and for holding one end of the capillary tube in the second reservoir. A gas connecting system connects the second reservoir to the pressure reservoir, the connecting system having a valve for venting the connecting system to the first pressure source and for blocking communication of the second reservoir with the pressure reservoir while venting to the first pressure source. The apparatus also has an insertion element for inserting the other end of the capillary tube into the sample reservoir and into the first reservoir. In the preferred mode, the apparatus includes a computer system for controlling the insertion element and the valve, so that when the other end of the capillary tube is in the sample reservoir, the valve permits communication of the second reservoir with the pressure reservoir for a controlled period of time for sucking the sample into the capillary tube. Additionally, in the preferred mode, the computer system causes the other end of the capillary tube to be transferred to the first reservoir after the sucking of the sample into the capillary tube. After the sample has been introduced into the capillary tube and the tube has been transferred to the first reservoir, the electrophoresis is begun.

An additional important feature of the preferred embodiment is that an automated temperature control system is provided which enables the temperature of the capillary tube (and hence the solvent/solute system) to be controlled during electrophoresis. This is particularly advantageous in that for many buffers, the pH is a strong function of temperature; hence temperature control is very directly pH control. Additionally the pH

can have a direct effect on the electrophoretic mobility and hence the separation efficiency.

In another embodiment of the invention, the capillary is prewashed and equilibrated to achieve substantially zero charge on the capillary wall, thereby essentially eliminating electroosmotic flow and substantially improving resolution.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows an apparatus according to the invention.

FIG. 1B is a section of a capillary as used in a preferred embodiment.

FIG. 2 is a table that illustrates the effects of capillary prewash on electroosmotic mobility.

FIG. 3 is a graph showing the results of FIG. 2.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

FIG. 1A is a partially sectioned illustration of a preferred embodiment of an automated capillary electrophoresis, henceforth CZE, apparatus according to the invention. In this preferred mode, the apparatus includes an environmental enclosure **11**, which has access openings (not shown), and feedthroughs of various kinds through the walls of the enclosure for elements that must be connected to elements outside the enclosure. Electrophoresis is accomplished within the enclosure in a capillary tube **13**, preferrably constructed of fused silica, such as is typically used for high sensitivity liquid or gas chromatography. One end of capillary **13** is immersed for the process in a buffer solution **19** held in a first container **21**, and the other end is immersed in a buffer solution **15** in a second container **17**. Buffer solutions **15** and **19** are typically the same solution, and many are well known in the art. Although in the prior art many different pH's have been used for the various buffers, depending on the particular experiment being performed, in this preferred embodiment, it has been found that for capillary electrophoresis that a relatively low pH is best. In the preferred mode, to achieve the best separations, the pH is adjusted to the point of zero electric charge of the buffer-capillary combination, i.e. the point at which there is no charge on the capillary wall. As will be discussed subsequently in more detail, the point of zero charge will vary depending on the buffer used and the pretreatment of the column. However, as a practical matter, typically a pH below about 2.5 will suffice. Also, as will be discussed subsequently, sometimes particular buffers are used which are temperature dependent, i.e. their pH varies strongly with temperature, or stated another way dpK/dT is relatively large.

FIG. 1B is an enlargement of capillary tube **13** in cross section. The internal diameter of the capillary, D1, varies for different kinds of samples and for other reasons. A typical value for D1 is 0.05 mm, and generally varies between zero and 200 microns The wall thickness of tube **13** is small enough that the tube is flexible and may generally be manipulated without breaking. Also, the small diameter allows for effecient heat transfer.

Second container **17** has an airtight top **25**. Capillary tube **13** enters the second container through a stopper **27** maintaining an airtight seal. There are two additional penetrations through top **25**. A hollow tubing **29** enters through stopper **31** and an electrode **33** enters through another stopper **35**. Stopper **35** is typically of an electrically non-conducting material. From electrode **33**, an electrical lead **37** goes to an electrical feedthrough **39** which allows an electrical signal or power to cross the wall of the enclosure without shorting to the enclosure. On the outside, electrical lead **41** goes to a terminal of a high voltage power supply **43**.

From the opposite terminal of power supply **43** another electrical lead **45** goes to another feedthrough **47**. Inside the enclosure lead **49** goes to an electrode **51** immersed in buffer solution **19** in first container **21**. With buffer solution and sample material in the capillary tube and the tube ends immersed in buffer solution in the two containers, power supply **43**, through the electrical leads, feedthroughs and electrodes, may be used to maintain an electrical potential across the material in the capillary tube.

The second container rests on a support **53** with an electrical insulator **55** between the container and the support. The insulator is needed if the container and support are electrically conductive. First container **21** rests on a moveable, sliding support **57** and an insulator **59**.

A detector **61** is positioned relative to the capillary tube to measure the results of electrophoresis in the capillary. Such detectors are well known in the art, and include for example an Applied Biosystems Model 783 Spectroflow UV/Visible Detector, which is a variable wavelength programmable detector that is specifically adapted for on-column detection. Electrical leads through feedthroughs **63** carry power and signals for the instrument. There may be more than the two leads shown.

When the electrophoresis process is complete on one sample, and another sample is wanted in the capillary for analysis, a new sample may be loaded without manual intervention or disturbing the environmental enclosure. A motor **65** powered by leads through feedthrough **67** and supported by bracket member **69** may be activated to turn lead screw **71**. Nut **73** is attached to member **75** With a clamp **77** securely holding tube **13**, so that turning lead screw **71** will raise and lower the tube by the distance between stops **79** and **81**. This distance is set to be sufficient for the lower end of capillary tube **13** to be raised above the rim of container **21**, and lowered again.

With tube **13** raised above the rim of container **21**, motor **83** may be activated by leads through feedthroughs **85** to turn lead screw **87** moving slide **57** along support **89**. A sample container **91** with multiple microvolumes such as **93**, arranged in a row in the container, is prepared in advance and placed adjacent to container **21** on slide **57**. Each microvolume may contain a sample to be analyzed Typical injection volumes range from 1 nl to 10 nl in this preferred mode, although other size samples could of course be chosen depending on the size of the reservoir used to hold the sample and the size of the column. By controlling motor **83** moving slide **57**, any one of the microvolumes of container **91** may be moved to be directly below the end of capillary tube **13**, which may then be lowered into the microvolume by control of motor **65**. Once a new sample is drawn into the capillary, the capillary may again be raised, container **21** returned to position, and the end of the capillary re-immersed in the buffer by lowering the tube.

To inject a new sample, while one end of the capillary is in one of the microvolumes of sample material, a relative vacuum is drawn in second container **17** by

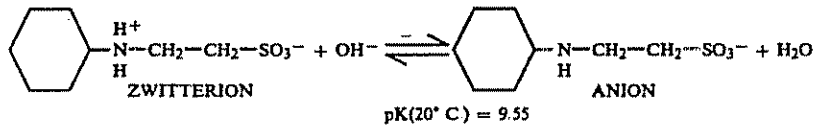
means of tubing 29 which exits the environmental enclosure. Motor 95 is controlled to rotate a three-way rotary valve 97 opening tubing 29 to vacuum reservoir 99. The reservoir is maintained at desired vacuum level by vacuum pump 101 through isolation Valve 103. A vacuum sensing gauge 115 with programmable signal points monitors the vacuum level in reservoir 99. The pump is powered by motor 105. Careful control of timing and vacuum level provides a very accurate method for drawing a predetermined amount of sample material into the capillary, as well as other benefits. As an example, using a pressure differential of 5.0 in. of Hg between the vacuum reservoir and the enclosure 11, with a 65 cm long fused silica capillary having a 50 micron inside diameter, a 2 second open time for valve 97 results in an injection quantity of 5 nanoliters of an aqueous solution.

Another important feature of the apparatus according to the invention is that the temperature inside the environmental enclosure 11 can be controlled. A heating element 107 is powered through feedthroughs 109 to provide heat, and a heat sensing element 111 monitors temperature through leads 113. As will be discussed subsequently, such provision for temperature control is very useful, since some buffers have a temperature dependent pH, and for those buffers pH can be controlled automatically by controlling temperature. Temperature control is also useful in the more general case since other kinds of variations are avoided if a uniform temperature is used throughout a separation. For example, viscosity and therefore mobility are most often strong functions of temperature, so that for reproducability, temperature control is required.

Power and control leads for all the electrical equipment associated with the apparatus of the preferred embodiment are carried by electrical conduit 121 to a control interface 119 which provides power terminations and switching of signals for control purposes. The control interface is connected to and manipulated by a computer 117 which can be pre-programmed so that critical parameters may be maintained and sequences of analyses may be performed automatically by the apparatus. For example, the vacuum level desired can be entered as control data, and the computer, through the control interface, monitors the signal from vacuum gauge 115 and opens and closes vacuum isolation valve 103 so that the desired vacuum level is closely maintained. As another example, the computer can be used to control the temperature inside the environmental enclosure by monitoring temperature sensor 111 and controlling power to heating element 107 as needed to maintain the programmed temperature. Also, the computer can be programmed to allow a sequence of analyses to be made, using the several samples preloaded into microvolumes in container 91, controlling the electrical devices in the required sequence The program may be set to run analyses on all of the microvolume samples, one-after-the other, or to allow for manual intervention and initiation between each analysis. Another important feature of computer control is that the computer makes it possible to reverse polarity of the capillary electronically. Hence, for solutes that are of opposite charge, one can reverse the direction of migration of solute particles and thereby use the UV detector at its fixed location. Details of the program structure for the computer are provided in Appendix A.

Control of pH

In capillary electrophoresis in free solution and in some gels, solutes with different charges (absolute) have different electrophoretic mobilities and can therefore be separated. Separation efficiency can be improved if the selectivity (i.e. the relative difference in electrophoretic mobility) between two or more solutes can be changed during the eletrophoretic run. One way to achieve this is to change the relative difference in the effective charges on the species to separated. In many cases the pH (or better, the pK) of the solution in the capillary, which is mostly buffer, determines the charge on solutes that obey the rules of acid/base equilibria. For example, for CHES (Cyclohexyl amino ethane sulfonic acid) in water, a chemical equilibrium is established which is dependent on the particular temperature, as indicated by the following formula:



at pH=9.55, 50% of the CHES is in the zwitterion form and 50% is in the anion form. By increasing the pH to 10.55, the anion (RSO_3^-) concentration will be ten times that of the zwitterion and by increasing it to 11.55, the equilibrium will be pushed almost completely to the anion side. At that point only 1% of the CHES will exist as a zwitterion. The anion will posess a certain electrophoretic mobility while the zwitterion being electrically neutral will not have an electrophoretic mobility. What this means is that at a pH of about 7.55, the CHES solute will have practically no electrophoretic mobility and at a pH of about 11.55, it will have nearly the mobility of the anion. Hence, by changing the pH of the solution, the mobility of a solute can be changed.

As indicated earlier, in many cases, the pH (or pK) of buffer solutions are a function of their temperature, and different buffer solutions have different temperature characteristics. (See CALBIOCHEM CATALOG, Table IV, Page 16.) By changing the temperature of the buffer in time or in space, different pH's in time and in space can be generated and thus the mobility of a species can be manipulated. In general, the pK of a solution is given by:

$$pK = pH - \log\{RSO_3^-\}/\{R^+SO_3^-\},$$

where $\{RSO_3^-\}$ corresponds to the concentration of the anion RSO_3^-, etc., and in many cases the pK has a strong temperature dependence. As an example of how to use this temperature dependence during the performance of a separation, suppose that a separation is to be performed on a solution containing three solutes, A, B, and C, and that the A and B separation is best performed at a first temperature T1 with A coming through first, and that the B and C separation is best performed at a temperature T2. The separation is run for a first time at temperature T1, until A is separated

from B and C, then the temperature is changed to T2 until B and C are separated. Similarly, more complicated temperature profiles can be used depending on the particular solutes being separated, for example continuous programming can be used if desired.

It should be appreciated by those skilled in the art that solutes that are to be separated may also obey the acid/base equilibria rules, and as a result also can change their degree of ionization With temperature. This solute effect will be superimposed on the pH change of the solvent (buffer) and hence, depending on the choice of buffer and solute combination, can provide an enhanced mobility difference, decreased mobility difference, or no change in mobility difference at all. Hence, various combinations of buffer and solute should be chosen to achieve the desired effect.

As a specific example of the effects of varying temperature, and hence pH, an experiment was conducted to investigate the relative electrophoretic mobilities of two proteins, Myoglobin (wsm) and Myoglobin (hh). A fused silica capillary was used having a 55 cm length to the detector, a total length of 70 cm, and an inside diameter of 0.050 mm. Using a 10 mM Tris-HCl buffer, and a 20 kV electric potential, the change in relative difference in electrophoretic mobilities (i.e. selectivity) of the two proteins was measured between the temperatures of 26.9° C. (pH=8.90) and 62.4° C. (pH=7.90), and was found to be minus 45%.

As described earlier, another important aspect of the invention in achieving a high selectivity, particularly in protein separations, is to eliminate charge on the capillary wall. The purpose is to eliminate electroosmotic flow, so that the column is not being swept during the run, thereby providing a longer separation time in the column (lower average velocity of the species to be separated), and hence better resolution. Also, by eliminating charge on the wall, positive ions (e.g. proteins) do not stick to the wall, unlike the typical case when the wall is negatively charged. One way to achieve zero charge on the wall is through control of pH. Generally, the electroosmotic velocity is proportional to the zeta potential times the applied electric field divided by the viscosity. The zeta potential describes electrostatic forces in the interfacial double layer between two phases and is, among others, a function of the differential adsorption of ions. When there is no electroosmotic flow, the zeta potential is zero, and there is no charge on the capillary wall. Hence, by measuring the electroosmotic mobility, i.e. the electroosmotic velocity divided by the applied field, as the pH is changed to achieve zero mobility, the point of zero charge on the wall can be determined.

An example of the effects on electroosmotic mobility resulting from pH changes and some surprising results from differences in capillary preparation are illustrated in the Table of FIG. 2, and in FIG. 3. In these experiments, a number of buffers were used in order to cover a wide range of pH levels, since as a general rule any one buffer has a relatively limited range of pH values over which it is useful. The capillary used was a fused silica capillary supplied by Polymicro Technologies, and had a length to the detector of 30 cm, a total length of 50 cm, and an inside diameter of 0.050 mm. The applied electric field used was 360 V/cm. Except for run 4, where the order of preparation was reversed to check for reversibility (i.e. steps 4, 5, and 6, preceeded steps 1, 2, and 3 below), the protocol for capillary preparation was as follows:

1. wash capillary with 1M NaOH for 3 minutes;
2. equilibrate with buffer for 5 minutes;
3. run with mesityl oxide as a neutral marker and measure elution time for the marker;
4. wash capillary with 1M HCl for 3 minutes;
5. equilibrate with buffer for 5 minutes;
6. run with mesityl oxide and measure elution time.

The buffers used were CAPS (i.e. 3-(cyclohexylamino) propane sulfonic acid), BICINE (i.e. N,N-bis (2-hydroxyethyl) glycine), MES (i.e. a sodium salt of 2-(N-morpholino) ethane sulfonic acid), and citric acid.

A graph of the electroosmotic mobility, shown in FIG. 3, illustrates dramatically the results of lowering the pH. Clearly, as the pH is lowered there is a sharp decrease in mobility indicating that the charge on the wall is quickly approaching zero. For NaOH treated tubes, a pH of below about 2.5 corresponds essentially to zero electric charge on the wall (the baseline noise is proportional to the current, so as a practical matter it is important to use a low current, and low pH buffer). Even more striking, however, is the effect of the prewash. Although NaOH is the quintessential strong base that is typically used for washing glass, it is clear that the use of a strong acid such a HCl is a much better prewash if the purpose is to eliminate charge on the capillary wall. For example, if the capillary prewash is performed with HCl, a pH of about 4.0 eliminates about 97% of the charge on the capillary wall, and produces an electroosmotic mobility that is even lower than that achieved using a pH of 2.5 if the prewash is with NaOH. Furthermore, it appears that the effects of the different prewashes are substantially independent of each other, since in run 4 where the order of the two prewashes was reversed, the results are substantially the same. As a practical matter, it appears that removing 95% or more of the charge on the capillary wall is important in performing high resolution capillary electrophoresis, regardless of the prewash that is used. However, it appears that eliminating that charge is much easier to accomplish, and allows use of a much higher and more easily attained pH level, if the capillary is prewashed with an acid instead of a base before the run.

It will also be appreciated by those skilled in the art that there are several ways to control the temperature of the solvent/solute system. For example, one way has already been described which uses a heater system for environmental chamber 11. Another approach would be to use one or more electrical heaters wrapped around the capillary tube, and another would be to use one or more pieces of insulating wrap on the capillary tube. Those skilled in the art will undoubtedly be able to think of other equivalent methods for controlling the temperature to effect electrophoretic mobility. Those skilled in the art will also understand that in some instances it may be preferred to not have all components inside the enclosure 11. For example, the detector sometimes may be located outside the enclosure along with the corresponding portion of the capillary where the UV detection is to take place. Such an approach Would facilitate service of th UV detector system. Also, instead of raising and lowering the capillary, one could raise and lower the sliding support to insert and remove the capillary from the sample and buffer reservoir. It should also be apparent that one could use electrophoretic media other than aqueous solutions, for example organic fluids could also be used, a specific example being acetonitrile.

What is claimed is:

1. A method of performing capillary electrophoresis comprising:

introducing an electrophoretic medium into a capillary;

introducing sample into the capillary;

applying an electrical field to the capillary to cause electrophoresis;

establishing a first temperature at a point in the capillary; and

changing the temperature at said point during electrophoresis to vary the pH as a function of time to cause differential separations in the sample.

2. The method of claim 1 wherein the step of changing the temperature comprises running the electrophoresis for a first period of time at a first temperature at said point and then running the electrophoresis for a second period of time at a second temperature at said point.

3. The method of claim 1 wherein the step of changing comprises changing the temperature at said point during electrophoresis according to a preselected temperature profile.

4. A method of performing capillary electrophoresis comprising:

introducing an electrophoretic medium into a capillary;

introducing sample into the capillary;

applying an electrical field to the capillary to cause electrophoresis;

establishing a first temperature for a length of the capillary; and

changing the temperature for said length during electrophoresis to vary the pH to cause differential separations in the sample.

5. The method of claim 4 wherein the step of changing the temperature comprises running the electrophoresis for a first period of time at a first temperature for said length and then running the electrophoresis for a second period of time at a second temperature for said length.

6. The method of claim 4 wherein the step of changing comprises changing the temperature for said length during electrophoresis according to a preselected temperature profile.

* * * * *

CERTIFICATE OF SERVICE

I hereby certify that on January 20, 2006, I electronically filed the foregoing document with the Clerk of Court using CM/ECF which will send notification of such filings, and hand delivered, to the following:

Josy W. Ingersoll
Karen L. Pascale
Adam W. Poff
Karen E. Keller
Young, Conaway, Stargatt & Taylor, LLP
The Brandywine Building
1000 West Street, 17th Floor
Wilmington, DE 19899-0391

I hereby certify that on January 20, 2006 I sent the foregoing document by Federal Express, next business day delivery, to the following non-registered participants:

Walter E. Hanley, Jr.
James Galbraith
Huiya Wu
Kenyon & Kenyon
One Broadway
New York, NY 10004

William G. James, II
Fred T. Grasso
Kenyon & Kenyon
1500 K Street N.W., Suite 700
Washington, DC 20036

Kelly E. Farnan

Kelly E. Farnan (#4395)
Farnan@RLF.com